EXPERIMENTAL CELL RESEARCH

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EDITORS:

T. Caspersson, Department of Cell Research, Karolinska Inst., Stockholm 60, Sweden

Honor B. Fell, Strangeways Laboratory, Cambridge, England

J. Runnström, The Wenner-Gren Institute for Experimental Biology, Stockholm, Sweden

Francis O. Schmitt, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Paul Weiss, University of Chicago, Chicago 37, Illinois

Ralph W. G. Wyckoff, Laboratory of Physical Biology, National Institute of Health, Bethesda, Maryland.

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STUDIES ON MITOCHONDRIA IN THE DEVELOPING SEA URCHIN EGG

TRYGGVE GUSTAFSON and PIERRE LENICQUE

The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

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The appearance of primary mesenchyme cells at the vegetal pole of the blastula initiates the period of visible differentiation of the main regions of the sea urchin larva. Subsequently, its structure becomes more and more complex by the elaboration of e.g. oral field, arms, stomodaeum, oesophagus, stomach, proctodaeum, coelom, secondary mesenchyme cells, skeleton, etc., cf. (15, 25). The morphological phenomena are parallelled by a series of chemical processes, such as changes in the amino acid composition of the larva (12), respiratory changes (25), and a strong rise in activity of a series of enzymes in the mesenchyme blastula (10, 11). It has been suggested (11) that the rise in enzymic activity expresses the development of units of associated enzymes, e.g. of mitochondria. These should constitute essential tools for production of proteins at differentiation. This hypothesis is supported by mitochondrial counts, as will be described in the present paper.

Treatment of the developing egg with LiCl during cleavage stages causes profound alterations in the development, the entomesoderm developing excessively at the expense of the ectoderm (9, 14, 27). In extreme cases, the whole larva may develop into an entomesoderm. The curves for enzyme synthesis of a Li treated culture of larvae generally ascend at a lower rate than do those of the control. This suggests that the ectoderm may have a stronger mitochondrial activity, i.e. a more elaborate mechanism of synthesis than the entomesoderm (11). One may, however raise the questions, whether there is a permanent difference in mitochondrial activity, or merely a difference with respect to its time-course. It is the scope of the present paper to examine these questions.

In previous papers of this series (9–12), the physiological background of the ecto- and mesentodermal development was studied by comparing normal larvae and larvae vegetalized by Li. Animalized larvae were also included in the present study. These were obtained by exposure to iodosobenzoic acid, according to the suggestion and preliminary observations of Runnström (46).

MATERIALS AND METHODS

An attempt was made to count mitochondria in homogenates in various media. Since preliminary experiments were not successful, the count of mitochondria *in situ* by aid of a phase microscope was attempted. Furthermore, a series of vital stains was tested. It now proved possible to make counts of the mitochondria by aid of an ordinary microscope of the highest quality (Zeiss HI 90 × apochromate, aperture 1, 40). Two to four drops of a 0.1 per cent stain solution was added to a two ml sample, the number of drops depending upon the density of the egg suspension. After 45 minutes, the eggs were placed on a slide, pressed under the coverslip, and fixed on the slide by traces of wax. The individual organ rudiments of the larvae selected for mitochondrial counts were still recognizable, and their animal-vegetal axes were parallel to the slide. The counts were made possible by inserting a network of 10 × 10 squares in the ocular. The slide was moved so that the different levels along the egg axis could be studied from the animal to the vegetal pole. This method is more clearly explained by fig. 1, from which appears that only a fraction of the mitochondria was counted.

In the first studies, the mitochondria were counted straight through the body wall. In the subsequent work the micrometer screw was not turned, but the mitochondria were counted in the focusing plane of the apochromatic lens. This plane has in fact also a depth dimension and constitutes thus a layer which is thinner than the body wall. Both ways of counting gave consistent results.

By pressing the coverslip, the normal and Li larvae of different stages were flattened to approximately the same dimensions. This was impossible in the advanced gastrula stage, owing to the different shape of normal and Li larvae. In an early gastrula stage, however, the invagination of the entomesoderm was reversed by pressing the larva, and counts could now be carried out without difficulty. As the intestine contains no mitochondria in this stage, a failing reverse of the invagination caused no serious errors. As the dorso-ventral organization of the larvae gradually manifests itself, the mitochondrial distribution within the ectoderm becomes less uniform. Furthermore, the appearance of arms etc. makes the orientation difficult, and also the interpretation of the results highly doubtful. Due to their shape, the Li larvae are more suitable for counts than are normal larvae. The Li larvae have thus been studied quantitatively also in more advanced stages. The mitochondria of the mesenchyme cells have not been counted. The primary mesenchyme cells are, however, free of mitochondria.

Young blastula stages were not studied, as a rule, since it was difficult to determine the polarity. Shortly before the mesenchyme appears, the vegetal pole is recognizable, however, by a slightly thicker blastula wall, by indentations from the blastocoel, and by a weaker stainability. Slight pressing of the blastula also causes release of cells, which presumably correspond to the future primary mesenchyme cells. When the vegetal pole was not recognizable, the mean of two counts along axes perpendicular to each other were applied. Since no clear mitochondrial gradient prevailed in this stage, this approximation may be allowed.

The vital stain selected for this study was nile blue sulphate, which is far superior to Janus green, dahlia and the other stains tested. Also the mitochondria of *Aplysia* are characterized by their exceedingly strong capacity of accumulating basic lipid-soluble stains (40). The color should not be added too rapidly, but dropwise, with a few minutes interval, otherwise crystals appear, and small blue oildrops also absorb the stain.

After staining, the mitochondria appear as violet-blue rods, often with slightly thickened ends. There is, however, a great variation in the thickness and length of the mitochondria, in the smoothness of their surfaces, and also in their movements. Also the color may vary somewhat. The phase contrast picture showed a typical mitochondrium to consist of two parts, more or less distinctly united by a bridge. Vitally stained mitochondria had approximately the same shape when observed in the phase contrast as when the apochromate was used. We assume that the stain partly adheres to a lipid-rich sheath surrounding the mitochondrium, cf. (31, 54).

Besides accumulating in the mitochondria, the vital stain also gives a diffuse color to the egg. The yolk granula and some other structures are faintly colorized as well. The diffuse staining of the cytoplasm disappears at the vegetal pole when the mesenchyme cells begin to appear. This has also been reported by other authors (6, 38).

Mitochondria appear to develop gradually from very small blue units. In view of this circumstance, it ought to be difficult to decide whether a particle should be counted or not. This difficulty seems easier to overcome than was expected, as different mitochondrial curves were surprisingly coincident.

Each of the single points recorded in the developmental curves corresponds to one larva (fig. 4, 5, 6, 7). The scatter is surprisingly small. In the Li curves it is more considerable, which is in agreement with a greater scatter in the morphological type.

In the gradient curves, each point represents the sum of the mitochondrial numbers of five consecutive rows of the network, in animal-vegetal direction. This gives essentially the same type of curve as if the counts of each single row was plotted, see figs. 2 and 3. In figs. 8 and 9 the squares were divided into five groups. The sum of each group represents the mitochondrial frequency at a certain level of the larva in the direction of the animal-vegetal axis. The extension of the different morphological regions was also recorded. This made it possible to arrange the free hand drawings of the larvae to fit with the gradient curves, cf. fig. 10 and further.

Preliminary experiments were performed on *Strongylocentrotus droebachiensis* and *Echinus esculentus* by aid of the phase microscope. The main studies were made on the shallow as well as the deep water form of *Psammechinus miliaris*. Here, both phase contrast and vital staining were applied. A single observation in *Echinocardium cordatum* was also performed. The results subsequently recorded refer to *Psammechinus*.

Li treatment was performed by adding isotonic Li solution to the culture. 5, 10, or 15 parts of LiCl solution was added to a final volume of 100 ml egg suspension. The exposure started at the end of the eight-cell stage and continued for three hours at 20° C. The eggs were then repeatedly washed with normal sea water and were further cultivated in normal sea water. The cultures were generally cooled to 13–14° C. for a few hours to retard differentiation, see legends of the figures.

Iodosobenzoic acid treatment was performed in order to animalize the larvae according to descriptions by Runnström (46). 2 ml 0.013 M solution was added to 25 ml of egg suspension at the eight-cell stage or a few minutes after fertilization. The whole development was pursued in the presence of iodosobenzoic acid.

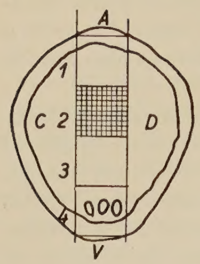


Fig. 1. Example of the use of the network for counting mitochondria; material: young mesenchyme blastula. The network is applied in the four levels 1–4 along the animal-vegetal axis (A–V), beginning with the most animal level. The actual position of the network in the figure is level 2. The number of mitochondria in each single small square of the network is noted in the protocol. When every small square is counted, the slide is moved until the network covers the next more vegetal level. The sum of mitochondria in each single row in the C–D direction is calculated.

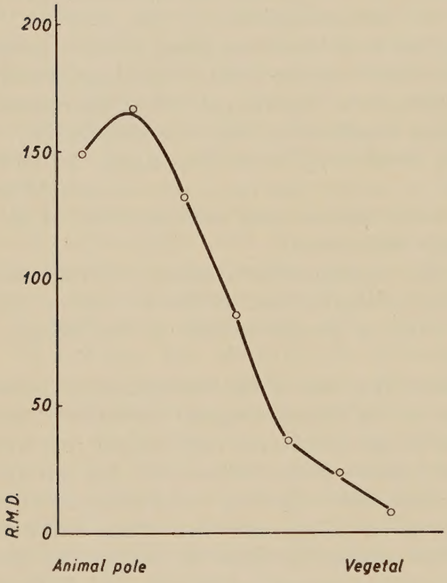


Fig. 2.

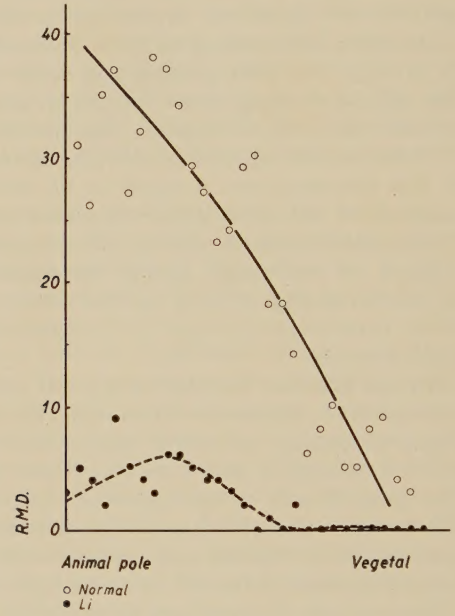


Fig. 3.

Fig. 2 and 3. Gradient curves showing the mitochondrial frequency in different levels along the animal-vegetal axis. The invagination of the archenteron material failed spontaneously; primary mesenchyme cells well developed; shallow form of *Psammechinus miliaris;* 9 per cent Li 2–6 hours after fertilization; 19–20° C; age 18 hours. *Fig. 2.* The number of mitochondria in each single row of the network plotted against the distance from the animal pole. *Fig. 3.* The number of mitochondria in five parallel rows of the network is plotted against the distance from the animal pole.

RESULTS

Early cleavage and blastula stages were not studied quantitatively. However, no great changes occur in the mitochondrial number. Subsequently, the mitochondrial frequency rises steadily, fig. 4–7. There is an apparent drop in mitochondrial number in later development. This drop is not representative of the whole larva, since the arm rudiments and ciliary bands are very rich in mitochondria.

After Li treatment, the mitochondrial number rises slower than in normal development, fig. 4–7. It continues, however, to rise after the apparent drop in the normal curve, but the bearing of the curves in advanced stages is doubtful. The differences between the normal and the Li curve may be great or small. This may be due to differences in the Li treatment or staining procedure. Furthermore, one has to take into account variations in the sea urchin material or individual error of the observers. In either case, the curves strongly remind of those for e.g. apyrase, glutaminase I, cathepsin II, and the dehydrogenases (11). The rise of the mitochondrial curves may, however, begin somewhat earlier than that of the curves referring to enzyme development.

As far as can be recognized from the present observations, the curves on the mitochondrial development as a function of time in animalized larvae do not differ considerably from the corresponding curves, referring to normal material. A different result might be expected if the larvae were transferred into normal sea water in later development.

The total number of mitochondria evidently shows a steady rise, apart from the apparent drop in the advanced normal gastrulae. This rise is more rapid in the animal halves of the mesenchymeblastula and gastrulating larva than in the vegetal parts. A gradient in mitochondrial frequency consequently appears in the mesenchymeblastula, see fig. 7 and further. It seems that this becomes more accentuated, due to the disappearance of mitochondria in the most vegetal part of the larva.

Mitochondria commence to appear in the intestine of the prisma larva. The entoderm of a larva of this stage immersed in a mixture of rhodamin B and nile blue sulphate presented very small and indistinct violet mitochondria. Very accurate observations with phase-contrast and nile blue staining of a 30 hour old prisma larva revealed a beginning appearance of mitochondria, apparently concentrated to the border of the entodermal lumen. The occurrence of light-refracting granula in the intestine of younger larvae has, however, been noted before the appearance of any stainable mitochondria. In the pluteus clear mitochondria, stainable with nile blue sulphate, occur in the oesophagus, stomach, and proctodaeum.

The gradient curves for the mitochondrial distribution in individual Li larvae do not change as rapidly from one stage to another in early development as do those of normal larvae, fig. 7 and further. The mitochondria-rich

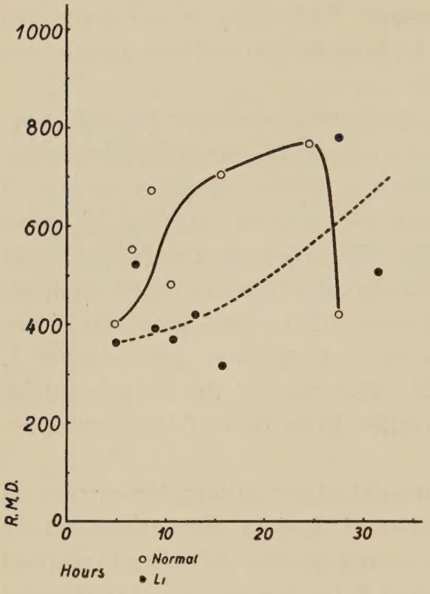
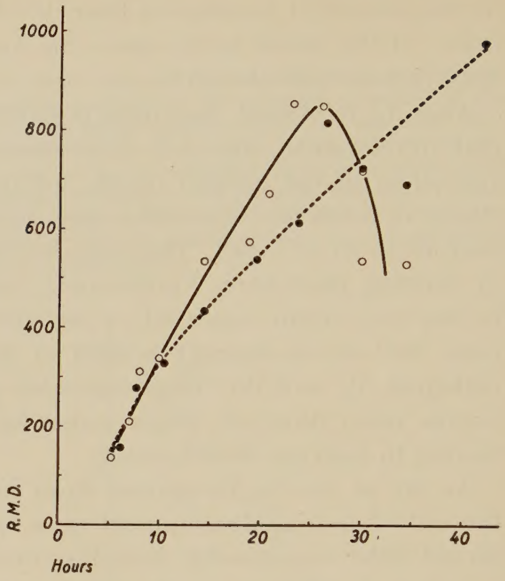


Fig. 4.



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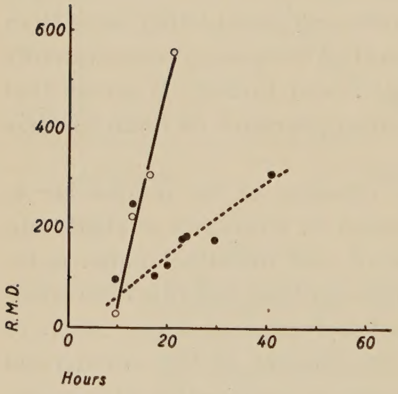

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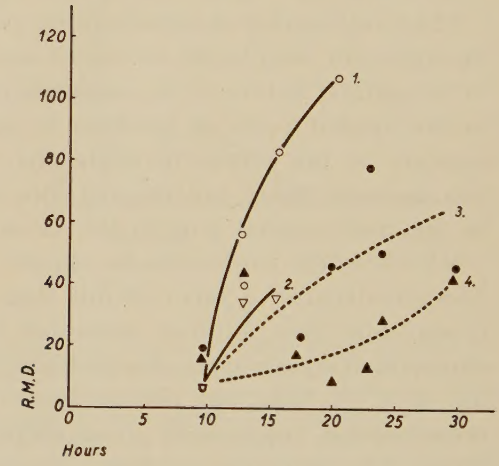
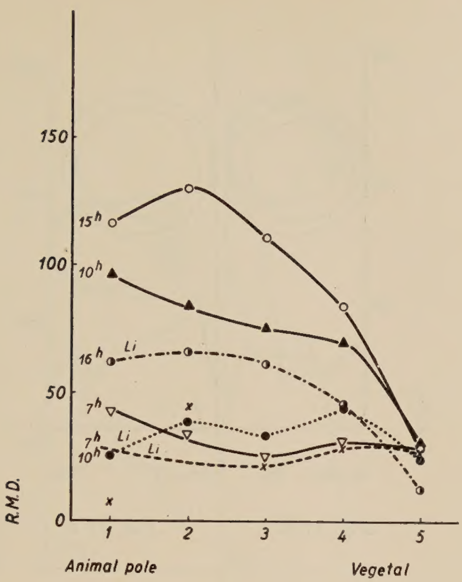


Fig. 7.

Fig. 4, 5, and 6. The relative mitochondrial number in normal and Li treated developmental stages as a function of the age of the stage; Li treatment between 3–6 hours after fertilization. *Fig. 4.* 20 per cent Li up to 13 hour sample, 15 per cent in the 16 hour sample, and 10 per cent in older samples; 19–21° C besides between 16–24 hours when the cultures were kept at 13–14° C; deep water form. *Fig. 5.* 15 per cent Li; 18° C besides between 15–24 hours when 13–14° C was used; deep water form. *Fig. 6.* 10 per cent Li; 18–22° C; shallow form.

Fig. 7. Mitochondrial counts in the most animal and the most vegetal 5 rows of the network in normal and Li treated material; shallow form; 10 per cent Li between 3–6 hours after fertilization; room temperature. 1. animal normal, 2. vegetal normal, 3. animal Li treated, 4. vegetal Li treated.



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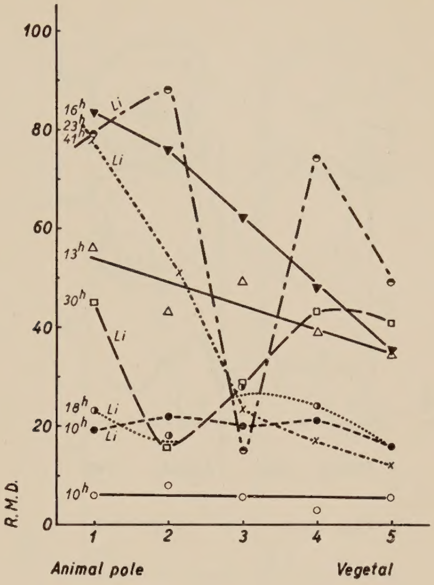


Fig. 9.

Fig. 8 and 9. Gradient curves showing the mitochondrial frequency in different levels along the animal-vegetal axis at different ages. Li curves dotted in different ways; age indicated to the left of each single curve; shallow form; 9 per cent Li 3–6 hours after fertilization; 17–20° C.

area is restricted to the most animal part of the blastula and to the early gastrula. The absolute mitochondrial density in this area may, however, be considerable. As development proceeds, the gradient curve gets complicated by the appearance of a secondary mitochondrial centrum in the developing entoderm-rudiment, fig. 9, 21, 23, 24, 26–29. This occurs much earlier in Li larvae than in normal larvae. The entodermal center becomes more and more pronounced, whereas there appears to be a decay in the ectodermal center, at least in most counts. In any case, the ectodermal mitochondria become immobile, thin or atypical, while the new entodermal center have thick, typical mitochondria which move rapidly. As development proceeds, the mitochondrial curve becomes more and more complicated, figs. 20, 21, 23, 24, 26–29, showing one maximum for the oesophagus and one for the proctodaeum. The proctodaeum mitochondria appear later than those in the other parts of the intestine. Sometimes the proctodaeum is not distinguishable from the ectoderm.

A coelom produced in a Li larva, fig. 31, no. 7, is always single, invaginated, and colorless, and apparently free from mitochondria, cf. invaginating

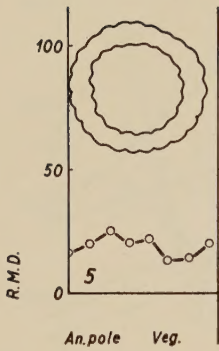


Fig. 10 a.

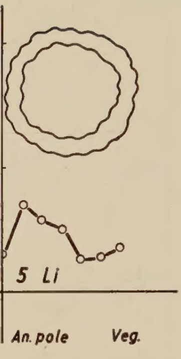


Fig. 10 b.

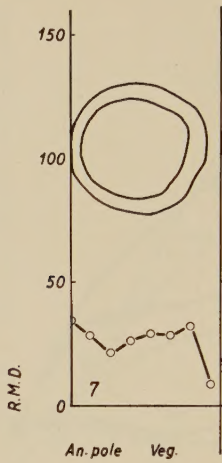


Fig. 11 a.

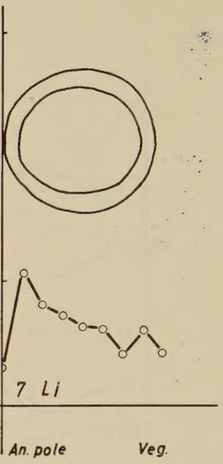
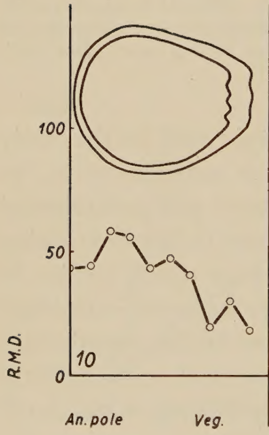


Fig. 11 b.



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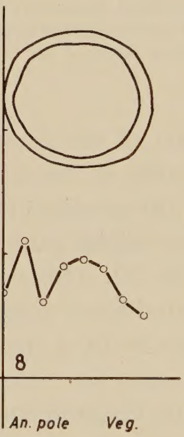


Fig. 12 b.

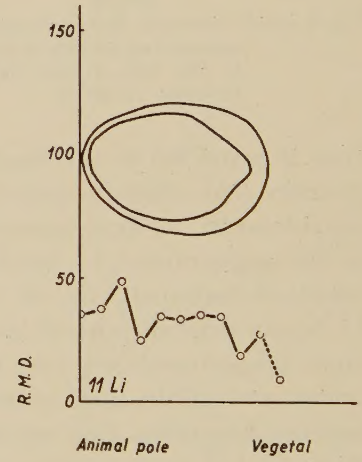


Fig. 13.

Fig. 10–30. Gradient curves showing the mitochondrial frequency in different levels along the animal-vegetal axis at different ages. Li and IBA denotes Li- and iodosobenzoic acid treatment, while curves without these indications refer to normal material; age in hours indicated in each single figure; temperature of cultivation about 17° C; deep water form; Li treatment between 3–6 hours after fertilization, 15 per cent in 10 b, 11 b, 13, 14 a, 16 a, 18 b, 19 a, 20, 21 a, 23 b, 24, 26, 10 per cent Li in 17 b, 23 a, 27, 28, 29. These examples chosen from different series of experiments, are typical.

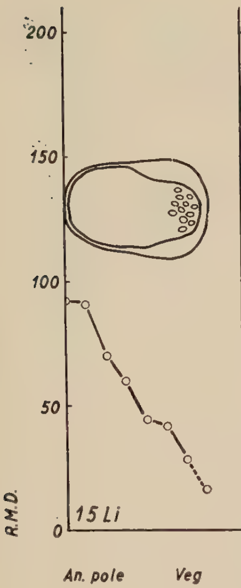
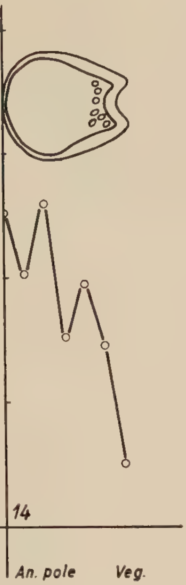


Fig. 14 a.



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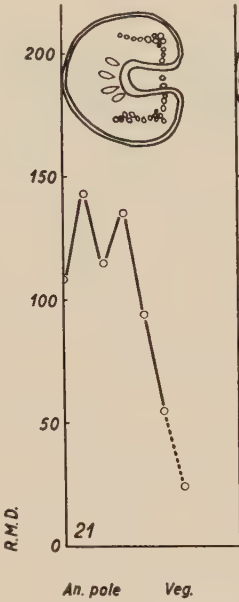

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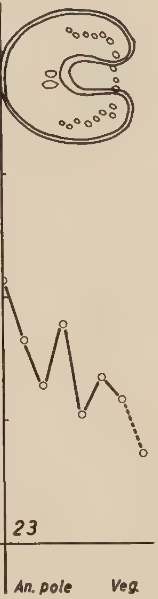

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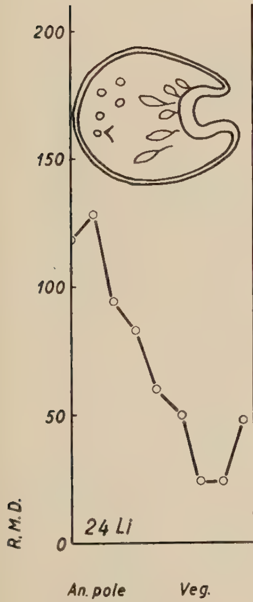

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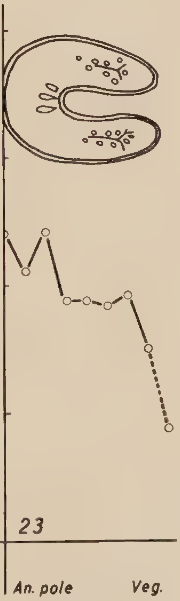

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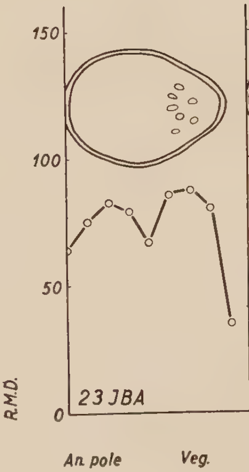
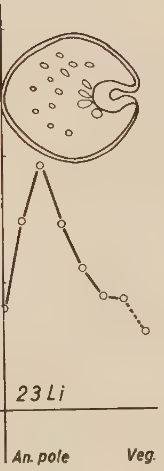


Fig. 17 a.



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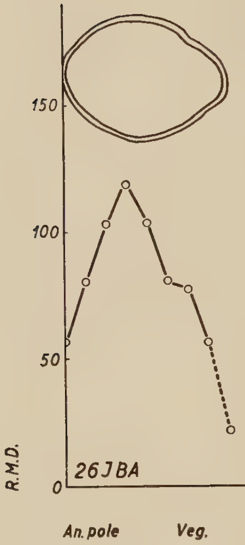
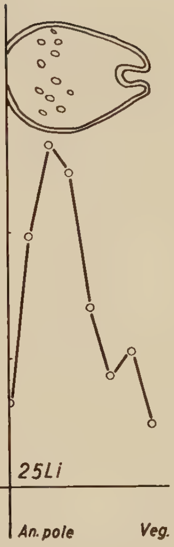


Fig. 18 a.



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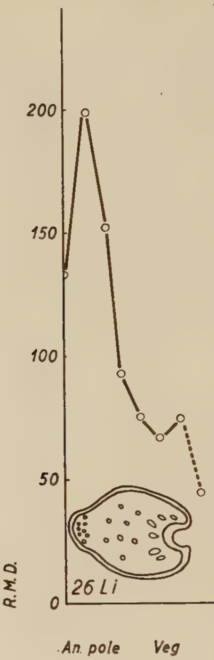

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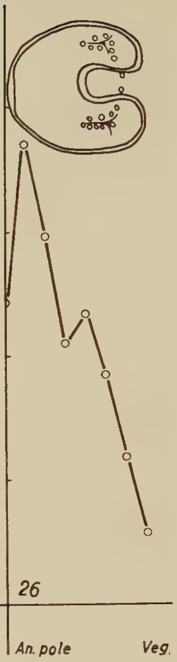

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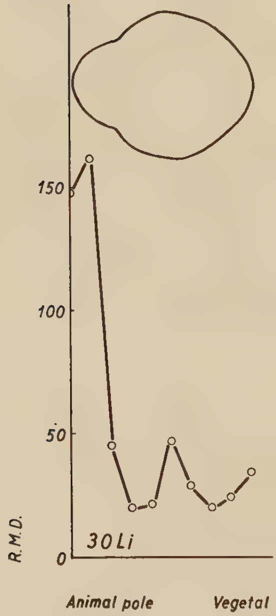

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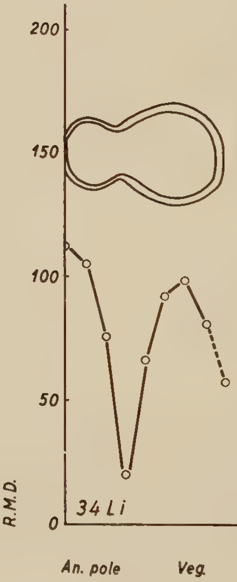

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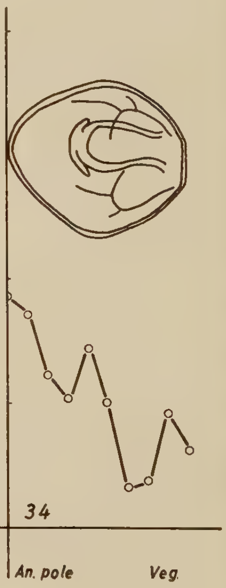

Fig. 21 b.

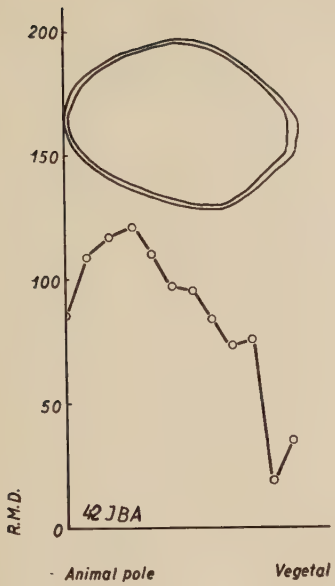

Fig. 22.

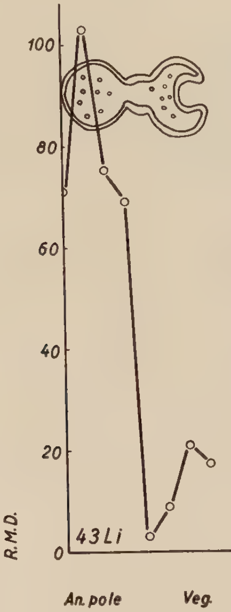

Fig. 23 a.

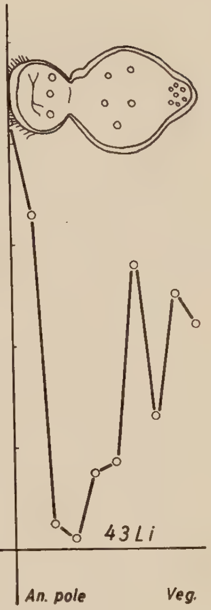

Fig. 23 b.

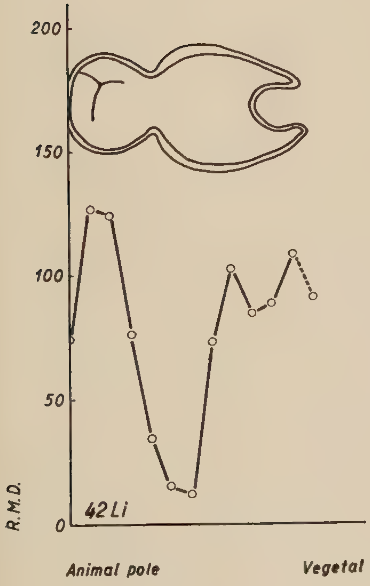

Fig. 24.

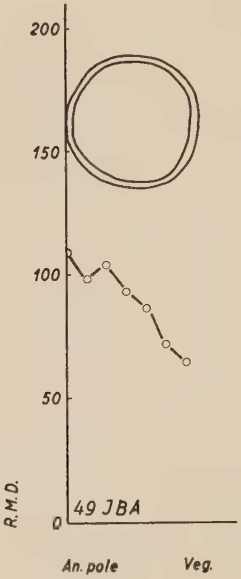

Fig. 25.

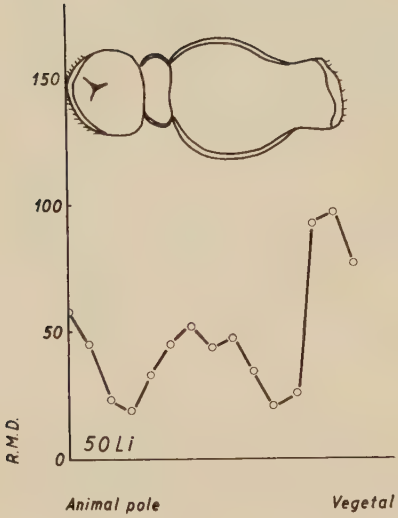

Fig. 26.

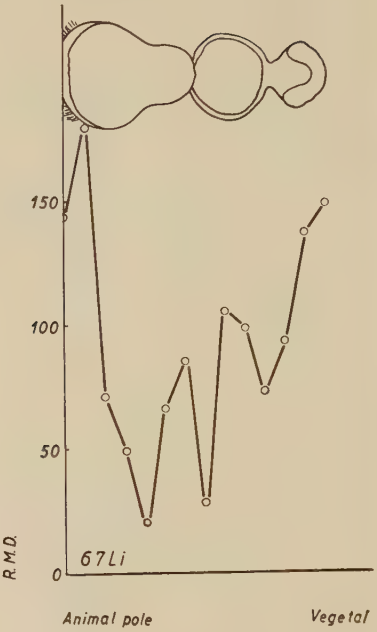

Fig. 27.

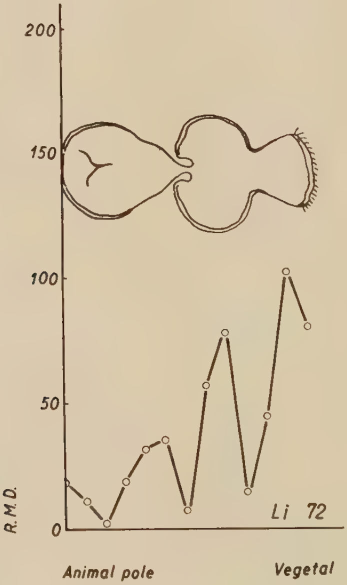

Fig. 28.

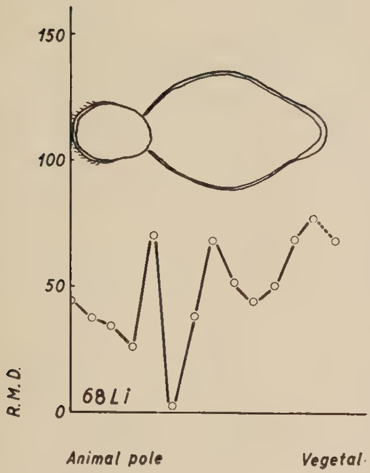

Fig. 29.

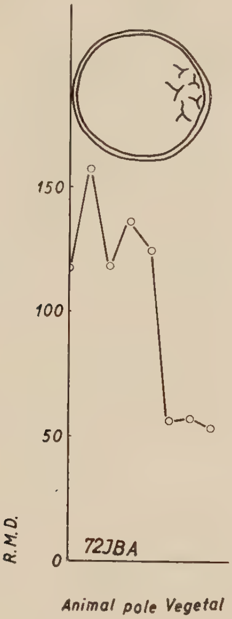

Fig. 30.

archenteron. If no coelom is formed, the oesophagus has a thick epithelium and the pigment granulae in the infiltrating mesenchyme cells are of more ectodermal type, cf. below. Occasionally, even a skeleton may be produced in the entodermic region. In one extreme case a few larvae with two typical ectodermal vesicles, connected by an intestine, were obtained in a culture, subsequent to five per cent Li treatment, cf. (24, p. 211–12).

The mitochondrial development in the Li larvae evidently demonstrates a rather constant succession, beginning with the ectoderm, continuing with the stomach, and ending with the proctodaeum. This succession has not been studied as yet in the intestine of normal larvae. It is of theoretical importance that the mitochondrial development is initiated earlier in the entodermal rudiment of Li larvae than in normal ones.

In animalized larvae (figs. 17, 18, 22, 25), the mitochondria-rich area is much more extended than in vegetalized and normal larvae. The total mitochondrial number is, however, not necessarily higher than in normal development. Thus, the lesser steepness of the gradient curve and not the absolute number of mitochondria seems to be essential. In animalized larvae with primary mesenchyme or even with skeleton, the mitochondrial gradient curve has a greater amplitude and thus a steeper slope than in more animal types. The morphological homogeneity is thus parallelled by a uniform mitochondrial distribution pattern.

In early Li gastrulae, most mitochondria in the vegetal part consist of thin rods. These rods appear to grow thicker as development proceeds and move very vividly. In older mitochondrial populations, however, immobile rods are frequent, and in still older ones the mitochondria are often small and atypical. Cases have been recorded, where mainly oil droplets are left in the ectoderm of old Li larvae. These types appear to represent a developmental series of mitochondria. Different parts of the larvae are generally characterized by different types of mitochondria, which is in agreement with the different age of the mitochondrial populations. This is especially evident in the Li larvae. Thus, the ectoderm demonstrates disintegrating mitochondria and oil drops in great amounts, whereas the proctodaeum is characterized by beautiful thick rods.

The appearance of mitochondria in an organ rudiment seems to be closely correlated with the phase of its most intensive differentiation. In young plutei, the arm rudiments and the ciliary bands surrounding the oral field are especially rich in typical mitochondria, whereas the mitochondria in the extended ectoderm is scarcer and thinner. The importance of mitochondria for differentiation is also illustrated by the increased mitochondrial number in the apical region, which may be considered as a suppressed extra oral side, cf. (17, 23).

The close connection between the localized development of mitochondria and the cell-stretching of the mitochondria-rich area appears from a comparison between the gradient curves, e.g. fig. 20 and fig. 31. The latter is drawn by aid of Abbe's drawing-apparatus.

Some of the mesenchyme cells contained mitochondria, but no pigment granula. These cells are connected by extended plasma bridges, and are arranged almost regularly in different larvae. They may therefore constitute primitive nerve elements. This suggestion is also supported by the fact that these structures in Li vegetalized larvae extend straight through the primary body cavity, from the animal pole to the contractile oesophagus. In a young gastrula, no mitochondria were present in the mesenchyme cells, except a few cells which are presumably "neuroblasts".

The mesenchyme cells were carefully and repeatedly studied, in order to reveal the presence of mitochondria. In a gastrula with beginning skeleton formation, the presence of one or two pairs of stained spheres per primary mesenchyme cell was demonstrated by means of rhodamin B, but no typical

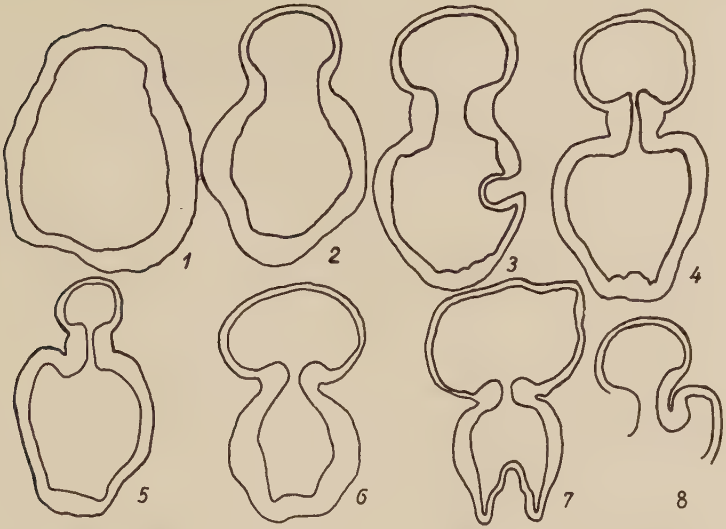

Fig. 31. Optical median sections of Li treated larvae in different stages of cell-stretching; Abbe's drawing apparatus; Li treatment as in Fig. 7; deep water form; 20° C; age of development in hours: 1: 26, 2: 29, 3: 33 $^1/_2$ with abnormal cell-stretching in a small part of the presumptive entoderm; 4–8: 33 hours.

mitochondria occurred. In secondary mesenchyme cells, however, pigment granula occur, as well as mitochondria.

In a gastrula with beginning skeleton formation, no pigment was apparent in the granula. Three hours later typical pigment was found. The increasing accumulation of pigment in older stages may also be studied in centrifuged samples of larvae. The color diffuses out if the larvae are washed in isotonic potassium chloride. It is, however, insoluble in acetone. Much more pigment is formed in Li larvae than in normal larvae.

On staining, the pigment granula accumulate nile blue sulphate and become black, and not blue or violet as the mitochondria. They also contain red unstainable components. In a single Li larva one can in fact study a series of transitions between purely black and purely red granula. Such transitions are e.g.: black granula with thin red borders, granula with thicker crescent-like red borders, and finally red granula with a big or minute black corpuscle in the periphery. The distribution of the different types of pigment granula in Li exogastrulae show interesting regularities, fig. 32. The dominantly black types are characteristic for the animal regions, and the more red types for the vegetal regions. In the most vegetal part the black component often gains in importance. In these cases the oesophagal epithelium

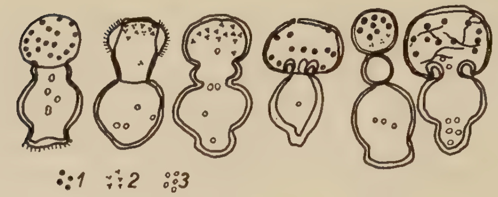

Fig. 32. Schematic figures demonstrating the distribution of different types of pigment cells in Li larvae; 1 denotes pigment cells with black granula, 3 denotes cells with red granula, while 2 denotes cells with granula of transition types; the material is the same as in Fig. 31; age about 3 days.

has the appearance of an apical plate, cf. above. The correlation between an ectodermal type of development and the production of "black" pigment granula is thus apparent. In the normal pluteus most of the pigment granula belong to the black type. The pigment cells are especially concentrated to the arm tips and to the apical region, where the ectoderm is characterized by high mitochondrial activity. In advanced starving plutei, the amount of red pigment is reduced. In larvae animalized by iodosobenzoic acid, neither pigment nor pigment cells are found.

DISCUSSION

The method of counting mitochondria *in situ* in the supravitally stained larvae presents a series of difficult practical problems. The two main questions formulated in the introduction appear however, to have been answered.

The developmental change in mitochondrial number closely agrees with the change in activity of a series of enzymes. As far as we know, both the enzyme and the mitochondrial curves show a rather constant and low level during early cleavage stages and a strong rise, beginning in the mesenchyme blastula. This rise is retarded after Li treatment. There is thus strong evidence that apyrase, glutaminase I, cathepsin II, and the dehydrogenases are localized in the mitochondria, as suggested in an earlier paper (11). The presence of liver glutaminase in mitochondria was recently demonstrated by a direct method (47).

The enzyme studies referred to have been performed with homogenates of whole eggs. Data on the distribution of the enzymes in the embryo were only obtained indirectly by comparing the curves of normal and Li treated material. The present results suggest that a study of the mitochondrial distribution also gives rather reliable data on the distribution of a series of enzymes in different parts of the embryo.

In the mesenchyme blastula and young gastrula there is an animal-vegetal gradient with respect to mitochondrial frequency. The gradient is not very distinct in early stages of development, when the total mitochondrial number is low. It gradually becomes more and more pronounced, as there is a strong rise in mitochondrial number within the animal region, whereas the number remains more constant in the vegetal region. The vegetal cells appear to produce inhibitors for the early mitochondrial development, cf. the experiments of Hörstadius (15) and the concept of Ranzi (36). The formation of inhibitors probably fails to occur in the presence of iodosobenzoic acid, and the animalized larva show a more uniform distribution of mitochondria. Following Li treatment, the inhibitor production should, on the other hand, be stronger than normally. The mitochondrial development is thus inhibited, and more so in the vegetal than in the animal regions. Summing up, the mitochondrial curves of animalized, normal and vegetalized larvae show characteristic differences in their slopes. The slopes of these gradients are probably controlled by a vegetal inhibitor, preventing a uniform mitochondrial distribution. Treatment with Li or iodosobenzoic acid is considered to interfere more or less directly with the formation of this inhibitor.

The Li inhibition of the animal type of metabolism may favour the inhibitor-producing metabolic type. The ability of a Li treated animal fragment, transplanted to an animal egg half, to inhibit the extension of the apical tuft (16) favours this concept. The type of metabolism which favours mitochondrial development probably decreases along an animal-vegetal gradient. It probably antagonizes the inhibitor producing type of metabolism which decreases in the opposite direction, cf. the double gradient system of Runnström (15, 27, 41–44).

The mitochondrial distribution in later differentiation cannot be described in a simple gradient curve, but progressively becomes more complicated with the development of new centra. Thus, the mitochondrial populations develop in a regular sequence along the egg axis. This can most easily be followed in Li larvae. It begins in the presumptive ectoderm and continues in the presumptive stomach. The intermediate part, the proctodaeum rudiment, is generally thick-walled and its mitochondrial population is sparse up to advanced stages. Finally, mitochondrial development also occurs here. This regular sequence may be elucidated by a series of well-known experiments. The most animal part which produces a ciliar tuft, inhibits the formation of the same structure in the adjacent levels. Removal of the most animal parts consequently permits the development of a ciliar tuft at a lower level

(15). The development of two separate cilia tufts at opposite poles in animalized larvae indicates that one rich differentiation prevents the close appearance of a second rich differentiation cf. (24, p. 211–12). The inhibition of one oral side on the development of a second oral side is another example of such phenomena. Constriction, stretching, and isolation experiments (17, 18, 23) indicate that these effects are due to substances produced by an intensely differentiating area. The latter may also drain off important diffusible substances from adjacent areas. It may be visualized that these compounds are related to developing and functioning mitochondria. The inhibitions from the developing ectoderm may thus finally decline, at first in parts distal to the ectoderm. This permits the development of a second wave of mitochondria, now in the presumptive stomach. This wave, on the other hand, suppresses the development of mitochondria in the adjacent presumptive proctodaeum. Summing up, the localized development of mitochondrial populations appears to be controlled by inhibitors, produced in regions of high mitochondrial activity.

The entodermal mitochondria in the vegetalized larva appear much earlier than those in the normal entoderm. This may easily be explained on the basis of the views presented above, since a small ectodermal part probably is less inhibitory than a normally sized ectoderm.

The same rules as apply to the Li larva probably hold for the development of the normal larva, with its more complicated oral structure. The dorsoventral organization present already in the unfertilized egg (8) may favour the development of the oral field.

Experiments with the eggs of *Ciona* (39) suggest an intimate relation between the distribution of mitochondrial germs and the subsequent production of structural proteins. When this egg is centrifuged, the oxidase and peroxidase positive regions coincide with the site of muscle differentiation. The development of an animal tuft of stereocilia in the sea urchin larva may be the result of an excessive production of structural proteins from the mitochondria. The cell-stretching in the ciliar tuft epithelium appears to be radial with respect to the blastula center, a process reminiscent of that occurring in the cells of the neural plate in the frog embryo. Whether radial or tangential, the cell-stretching may depend upon the action of oriented fibrous molecules, produced by mitochondria; cf. the concept of Waddington (52). A constant succession in the development of mitochondrial populations may thus cause a constant sequence of cell-stretchings. These gradually deform the thick and uniform blastula wall, thereby giving the larva its final external shape. This concept is supported by the observed parallelism between mitochondrial development and cell-stretching. The importance of mitochondria in differentiation is also exemplified by the studies on the "neuroblasts" mentioned above. A gradual consumption of mitochondria may take place as differentiation proceeds.

The concept outlined above is best illustrated by the development of a Li vegetalized larva. Here, the extent of the ectoderm appears to be determined by the extension of a mitochondrial population of a certain minimal density at a certain critical time. When the mitochondria start producing proteins for cell-stretching, a certain part of the thick blastula wall begins to expand. This part constitutes the presumptive ectoderm. It is separated from the presumptive entoderm by a rather sharp border. In the same way, the localized development of mitochondrial populations in the entoderm is followed by a constant succession of cell-stretchings. If the mitochondrial frequency is about the same in each level along the egg axis, as may occur subsequently to iodosobenzoic acid treatment, the whole blastula wall is stretched simultaneously and a single ectodermic vesicle is produced. In a normal larva, the critical border in mitochondrial frequency lies within the vegetal hemisphere of the blastula. In this case, the expanding ectoderm cells may exert a pressure which contributes to the mechanism of invagination.

When the primary mesenchyme appears, the mitochondria in the most vegetal parts of the blastula disappear, at the same time as the diffuse staining of the cytoplasm of the most vegetal cells vanishes. Neither vital staining nor phase contrast microscope revealed any typical mitochondria in the primary mesenchyme or in the invaginated archenteron. The disappearance of the coloration, also demonstrated with a series of other basic stains (38), depends upon a reduction which is reversible in the case of nile blue sulphate (38). The mitochondria are evidently not responsible for the maintenance of this reduction, since it is limited to the portions poor in mitochondria, in *Paracentrotus* as well as in *Aplysia* (40). The reduction is confined to the hyaline cytoplasm. Janus green stained mitochondria may remain colored, even when the cytoplasmic stain is reduced (40).

Is is tempting to suggest that the mitochondria present in the vegetal parts of the early blastula are consumed for morphogenetic purposes. The utilization of the cortical granula for the formation of the fertilization membrane presents an analogous example (45). The suggestion is also supported by the observation of Pease (32) that substances required for gastrulation in *Dendraster* are displaceable by centrifugation of the unfertilized egg. Furthermore, Lindahl (22) showed that substances active in cell-stretching are displaceable in the same way in *Psammechinus*. It appears as if the primary invagination were dependent on structure proteins derived from the vegetal mitochondria. When the first part of the invagination has occurred, the rest of the presumptive entoderm may be forced to continued invagination as a result of ectodermal cell-stretching. In the third phase of the entodermal development, the passively invaginated material may be stretched by the structure proteins produced by the intrinsic mitochondria of the entoderm. The passive role of the vegetal parts in gastrulation has also been suggested by Lindahl (22), on the basis of centrifugation experiments in *Arbacia*. Furthermore, as shown by Runnström (41, 42), the vegetal material is invaginated even if it is damaged with nile blue sulphate.

There is an apparent lag between the Li treatment in early cleavage stages and its effects on the enzyme synthesis and the mitochondrial development. There is, however, an immediate reversible inhibition of the respiratory rise, but no inhibition of the actual intensity (25). One of these writers (9) suggested that the respiratory rise might be associated with the Li sensitive growth of a particle population, the density of which determines the rate of protein synthesis at differentiation. This concept agrees with a number of different experiments and interpretations. Experiments of Harvey (13) indicate that mitochondria may be produced from non-mitochondrial precursors. Chantrenne and Jeener (5, 19) give evidence for the gradual development of mitochondria from smaller particles. The present observations show that minute stainable elements occur before typical mitochondria in the presumptive entoderm of a vegetalized larva. Furthermore, there is a discrepancy in the mitochondrial counts of larvae which are stained with different intensity. In a series with strongly colorized larvae, an apparent mitochondrial rise begins at hatching, whereas in other cases many enzymes, like the mitochondria, show a quantitative rise only in the mesenchyme blastula stage.

The gradual development of particles of different size was demonstrated by means of electron-microscopy on the *Tubifex* egg (20). It would be interesting to know, whether the differences in size between the particles of different regions are permanent, or if only the growth rate of the particles differs.

The point of attack of Li is not known. It may block certain chemical reactions, e.g. in the phosphate metabolism, by competing with potassium on the surface of an enzyme molecule (24), cf. (27, 28). The action of Li on the physical state of certain proteins was emphasized by Ranzi and collaborators, cf. (9, 35). It is also too early to settle the intracellular localization of the primary disturbances. A final effect on the building up of the mitochondrial population does not exclude a primary action in the nuclei, the immediate role of which has been demonstrated for the mitochondrial and cellular metabolism (1, 2, 4, 30, 33). Data in the literature indicate an effect of Li on the structure of the nucleus and its position in the cells (37, 51). Runnström's recent studies on the effect of iodosobenzoic acid also emphasizes the role of the nucleus (46). It is difficult, however, to decide whether the observed nuclear changes are the cause or the consequence of the cytoplasmic changes.

There is a continuous variation in the structure of the pigment granula, from apparently basophilic granula to red pigment spots, unstained with nile blue sulphate. The clear correlation between the type of the granula and their site along the egg axis suggest that factors influencing the mitochondrial development also act upon the development of the pigment granula. These granula may represent different stages of development of a certain mitochondrial type.

When the differentiation starts, the animal development is characterized by the intensive growth of a mitochondrial population. The vegetal parts in early differentiation may be characterized by protein combustion (12, 24), sulphate esterification for detoxification of aromatic amino acids (24), and pigment formation, possibly interrelated with the esterification (24). Lindahl demonstrated the presence of a phenolsulphatase, which may catalyze the reaction of sulphate with aromatic waste products (24). This will characterize certain vegetal derivatives. The sulphatase activity is constant throughout development (11). The intracellular activity of the enzyme may, however, vary, as phenolsulphatase is sensitive to redox-conditions and is activated by various reducing agents (50). The sensitivity of pigment production to iodosobenzoic acid suggests that SH-groups be involved in the process. The sulphatase may possibly be activated by the reducing conditions appearing at the vegetal pole of the late blastula (6, 38). It could also activate the cathepsin II of the mitochondria in the direction of proteolysis, so that the mitochondria dissolve. Mitochondrial development and protein synthesis on one side, and pigment synthesis, sulphate esterification, and protein degradation on the other, may thus constitute two alternate competitive types of metabolic pattern in the developing echinoderm egg.

Certain types of spontaneous liver tumours, induced or spontaneous, have a lower amount of mitochondria nitrogen than has normal liver (48, 49). Carcinogenic substances are known to accumulate and probably disturb the activity of the chondriosomes (53) as does Janus green (21). Carcinogenic substances and Janus green are known to interfere in regeneration and embryogenesis (7, 34, cf. 26). A further knowledge of the two metabolic types operating in echinoderm development and of the ensuing mitochondrial distribution may thus contribute to the elucidation of the cancer problem.

Mitochondria with different synthetic activity probably occurs also in the sea urchin egg. The pigment granula may be interpreted as a specialized type of mitochondria. Some of the mitochondria of the pluteus stomach also look aberrant. Much work remains to be done before this question will be settled. The study of the mechanism governing the development and distribution of various types of mitochondria is of fundamental importance to developmental physiology, as is also emphasized by Brachet (3).

SUMMARY

The relative number and the distribution of mitochondria in different developmental stages of the sea urchin egg has been studied by aid of the phase microscope and by vital staining with nile blue sulphate. The results have been interpreted as follows: The primary determination of the germ layers corresponds to a lithium-sensitive development of mitochondrial precursors. These gradually transform into mitochondria during the period of visible differentiation. The mitochondria contain a series of enzymes involved in the synthesis of e.g. fibrous proteins active in cell-stretching and in the formation of the apical tuft. A uniform distribution of mitochondrial germs in the early blastula is followed by a uniform cell-stretching, giving rise to a thin-walled ciliary blastula, i.e. an animalized larva.

Differentiation becomes heterogenous by the interference between the two main regions of the germ, characterized by different, mutually antagonistic metabolic types. One type, showing an animal-vegetal gradient of intensity, *favours* the development of mitochondria. The type with an opposite gradient *produces inhibitors* for mitochondrial development.

Iodosobenzoic acid, reacting with SH-groups, checks the vegetal inhibition, causing a uniform mitochondrial development and cell-stretching. Lithium treatment, favouring the inhibitory type, reduces the extension of the first mitochondrial population and the first wave of cell-stretching. A transient inhibition from very active mitochondrial centra of the germ checks the mitochondrial development in adjacent parts. A gradual development of mitochondria in these parts after recovery causes a second wave of cell-stretching and secondary inhibitions of adjacent parts and so on, until the whole thick blastula wall is segmented into distinct larval organs. A localized development of mitochondria thus causes a localized cell-stretching of the thick blastula wall. Development becomes further com-

plicated by the interference of a dorsoventral structure already present in the unfertilized egg. The vegetal metabolism may transform mitochondrial precursors into different types of pigment granula. Gastrulation is considered to be initiated by the consumption of preformed mitochondria. In the subsequent phase, the archenteron is more or less passively invaginated through the pressure evolved by the expanding ectoderm.

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GROWTH PROMOTING FACTORS IN DEVELOPING SEA URCHIN EGGS

TRYGGVE GUSTAFSON, MAJ-BRITT HJELTE
and INGRID HASSELBERG

The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

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Interactions between different areas of the germ constitutes a main problem in developmental physiology. In many cases, these interactions may be effected by chemical agents spreading from one embryonic region to another, as in the neural induction, cf. (1). Fragments of amphibian blastulae from lethal bastards, or of gastrulae which have been submitted to thermal chocks may develop normally when transplanted to a normal host. This evidently also depends upon the supply of diffusible agents from the latter (2). Extensive studies on sea urchin development also give evidence for the interplay of diffusible factors (12, 13).

Brachet suggests that factors active in neural induction spread like a virus infection (1). The present work deals with low-molecular weight substances, extractable by various means from different developmental stages of sea urchin eggs. Such compounds may act as mediators of certain interactions in development. The influence of these substances on the growth rate of *Lactobacillus casei* has been studied. This organism was chosen as test object, due to its growth response to certain peptides (4, 16).

MATERIAL AND METHODS

The material for the present study was the same as that used for the assay of amino acids (7), namely rapidly frozen, lyophilized, normal and lithium-treated developmental stages of *Paracentrotus lividus*. The motivation for a comparison between normal and lithium-vegetalized material was given previously (3, 6, 7, 8). The material had been kept at 4° C in a vacuum desiccator over anhydrous silica gel for 3 to 4 years.

250 mg lyophilized eggs were vigorously stirred for 30 minutes by aid of an air-driven high-speed homogenizer with 20 ml 75 per cent alcohol, sand, and glass-pearls. The mixture was then boiled with reflux for 20 minutes. Upon centrifugation the extract was decanted through a Munktell 00 filter paper, and the residue was refluxed with 10 ml alcohol for 10 minutes. The extracts and alcohol washings of tubes and

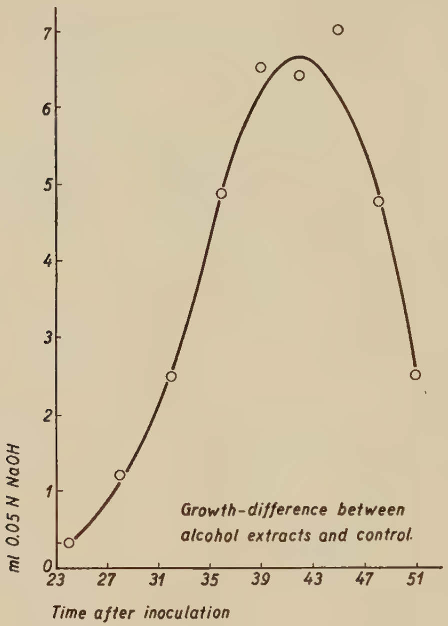

Fig. 1.

filter paper were combined and evaporated to dryness *in vacuo* on a boiling water bath. The lipids were extracted twice from the residue with ether at 29° C for 30 minutes, each time with 25 ml. The lipid-free residue was dissolved in 50 ml M/12 pH 6.81 phosphate buffer. Alternately, 150 mg eggs were extracted with 7.5 ml boiling water for 2 minutes. Upon cooling, the material was homogenized for 10 minutes. Thereafter 1.5 ml tungstic acid (5 parts 10 per cent Na-tungstate + 7 parts 0.6 N sulfuric acid) was added and homogenization continued for 5 minutes. After centrifugation, 6 ml neutralized extract was diluted to 20 ml with buffer, as below. On extraction with water, the boiled sample was instead homogenized for 15 minutes upon cooling. After centrifugation (15 minutes, 8 000 r.p.m.), a 5 ml neutralized aliquot was diluted to 20 ml with a pH 6.81 phosphate buffer, the final phosphate concentration being M/12. The extracts were always subsequently autoclaved for 30 minutes at 104° C in order to destroy glutamine, which otherwise stimulates growth in *Lactobacillus casei* (10, 5). They were kept overnight at room temperature and stored in a refrigerated box until use. For the extractions, equal parts of three different preparations of the same stage were mixed. The extracts correspond to about 5 mg lyophilized eggs per ml. The amounts of extracted egg material were, however, in each case corrected so as to correspond to the same amount of total Kjeldahl nitrogen. This is fairly constant up to at least the 30 hour stage (6, 7).

The medium used for the microbiological assay (11) was supplemented with a series of compounds, in order to minimize the influence of the same substances as present

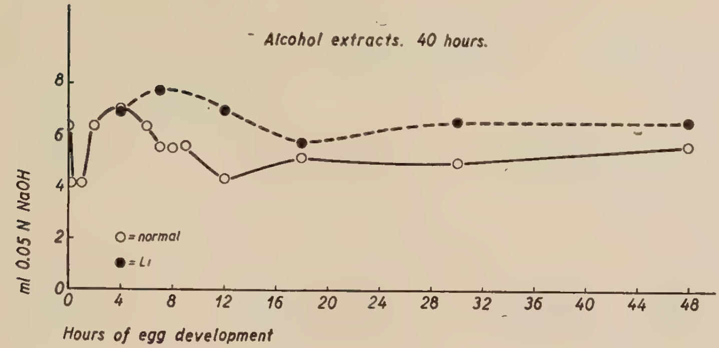

Fig. 2.

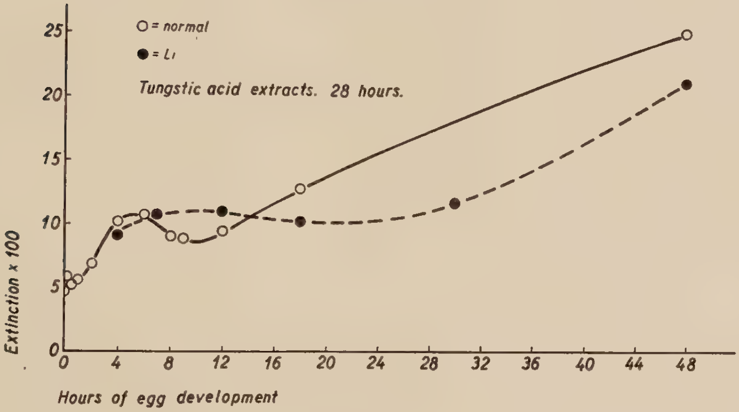

Fig. 3.

in the egg extracts. The compounds were: 2 mg cozymase, 2 mg β-alanine, 25 mg ascorbic acid, 2.5 mg choline chloride, 5 mg glutathione, 1 mg adenosine, 1 mg guanosine, 1 mg cytidine, 1.7 mg inositol, 0.1 mg oleic acid, 10 mg Na-malate, 10 mg Na-succinate, 10 mg Na-fumarate, and 0.5 mg lecithin. The amount of K_2HPO_4 used was changed to 0.21 g. 10 mg thymonucleic acid from *Paracentrotus* sperms, and 10 mg yeast nucleic acid were also added. Each of these was hydrolyzed in 5 ml 0.1 N HCl at 90° C for 10 minutes. Strepogenin (14–16) (kindly supplied by Dr. E. Kodicek, Cambridge), prepared from crystalline insuline, or vitamin B_{12}, added to the medium, do not eliminate the growth promoting effects of the extracts. In some alcohol-series thymidine (17) was also added, but this did not influence the main results. The sample was, however, not chemically tested.

One ml extracts of different developmental stages were autoclaved in pyrex tubes for 20 minutes at 120° C. The blank tubes contained buffer instead of extract. The medium (diluted with glass-distilled water 25:15) was autoclaved separately. Upon

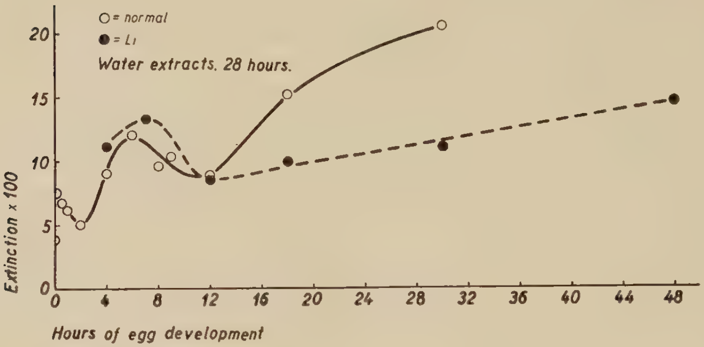

Fig. 4.

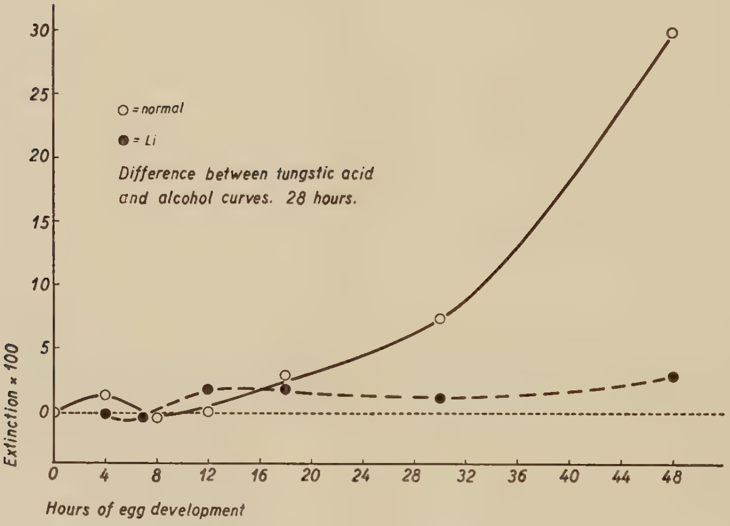

Fig. 5.

cooling, the medium was inoculated with a three times washed 18–22 hour bacterial culture, grown on "Difco micro inoculum broth." The density of the bacterial suspension was the same in different series as demonstrated by turbidity measurements of the inoculum. 4 ml of the inoculated ice-chilled medium was added to each single tube by aid of a syringe under sterile conditions. The tubes were chilled in an ice bath. Upon inoculation the tubes were transferred into a 37° C constant water bath, and incubated for different times. The first sample for examination was removed immediately after inoculation. The second sample was taken out after the first visible growth had appeared. This usually occurred after about 25 hours in the alcohol series, but after 20 hours in the water and tungstic acid series. The samples were then removed with e.g. 1 $^1/_2$–3 hours intervals. The tubes were chilled in an ice bath and subsequently kept in a refrigerator box, until the growth was estimated. This was made by aid of electrometric lactic acid titrations in earlier alcohol-series. In subsequent experiments, the turbidity was measured by aid of a Spekker spectrophotometer with a 0.5 cm cell and the H 508 filter. In the turbidity measurements, the experiments were finished at an earlier phase of bacterial growth than in the titration experiments. The readings were linear to the density of the bacterial suspensions in the range studied.

The growth curves were plotted on the basis of the samples removed at different times after inoculation. Each single developmental stage is represented by an individual growth curve. The difference in growth between an extract curve and a blank curve was used as the measure of the growth stimulating capacity. In earlier periods of incubation this difference was minor but gradually increased, attained a maximum and finally decreased, cf. Fig. 1. The more the growth curve bends off, the steeper is the drop of the difference curve. The growth stimulation of different extracts was compared at a time of incubation when the growth was approximately linear. If the comparison was performed when the growth difference between extract tubes and control tubes was maximal, or before that time, essentially the same results were obtained. The amplitude of the egg developmental curves depended upon the time chosen for comparison.

RESULTS AND DISCUSSION

The growth stimulating effect of the alcohol extracts after 40 hours' incubation, Fig. 2, shows a maximum in early cleavage stages and a minimum at 12 hours, followed by a certain rise. In lithium-treated material, the maximum is definitely shifted to an older stage, and the level is higher than normally throughout development. Tungstic acid and water extracts give curves very similar to each other and resemble the alcohol curve up to 12 hours after fertilization, Fig. 3, 4. At this stage, however, there is a considerable rise. This rise is strongly inhibited in lithium-treated material.

One gets the impression that the component, soluble in 75 per cent alcohol, causes the early maximum of the water and tungstic acid curves. After 12 hours an alcohol-insoluble component appears. This interpretation is supported by an experiment in which alcohol and tungstic acid extracts tubes were run simultaneously under the same conditions, and the difference in growth was plotted against the stage of egg development, Fig. 5. In this experiment the early maximum fails.

The growth promoting components may be separated by aid of paper partition chromatography with phenol-water. The fractions were localized on the chromatogram by aid of microbiological tests of water extracts from the different zones. There are two main fractions of growth promoting substances in 75 per cent alcohol extracts of a 4 hour cleavage stage, one with the mean R_F value 0.5 and another, quantitatively less important, with R_F 0.9–1.0. In tungstic acid extracts from an 18 hour gastrula, the latter component is quantitatively more important than in the alcohol extracts above. Acid hydrolysis liberates numerous amino acids from the slow fraction as well as from the rapid one, simultaneously destroying their growth promoting effect.

The water and tungstic acid curves resemble the quantitative developmental changes in the "peptide containing fraction" obtained as a difference between Kjeldahl N and Van Slyke amino N (7, Fig. 5). In both cases the curves reach an early maximum, a minimum occurs at 12 hours and is followed by a subsequent rise. It is tempting to suggest that the component before 12 hours mainly corresponds to short peptides soluble in 75 per cent alcohol, whereas the component after 12 hours corresponds to long alcohol-insoluble peptides. These peptides may form consecutive stages in protein synthesis from amino acids. When the mitochondrial activity becomes intensified (8), the accumulation is gradually shifted from short to long peptides. In lithium-treated stages where mitochondrial development is retarded, the early type of accumulation persists throughout development. The alcohol-insoluble component may, however, constitute a breakdown product of proteins, since a cathepsin II activity appears in advanced stages. In either case the strepogenin-like effect of the compounds may be of very great biological importance.

Important changes in the peptide fraction was also demonstrated in *Drosophila* development (9). Apart from strong quantitative differences, one peptide disappears completely at a critical stage of development, whereas another peptide appears. A great variation during the development and between different species might be expected for intermediates in processes of synthesis, since their accumulation is dependent upon the rate of formation, as well as on the rate of consumption. The accumulation does not in itself give information about the turnover rate of a substance. A low turnover rate may, in fact, result in an accumulation. The study of accumulation is, however, of importance for the understanding of chemical interactions. Such interactions may involve the inhibition and promotion of growth by closely related peptides, cf. (14, 15, 16).

SUMMARY

Alcohol, water and tungstic acid extracts were assayed on growth promoting substances in *Lactobacillus casei*. The developmental stages differed in

their content of these componds, and lithium-treatment of the germs favoured the accumulation of one component, whereas it inhibited the accumulation of another.

The authors wish to express their cordial thanks to Professor John Runnström for the great interest he has shown in our work.

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THE SUBMICROSCOPIC ORGANIZATION OF VERTEBRATE NERVE FIBRES

AN ELECTRON MICROSCOPE STUDY OF MYELINATED AND UNMYELINATED NERVE FIBRES

H. FERNÁNDEZ-MORÁN[1]

Institute for Cell Research, Karolinska Institutet, Stockholm

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Preliminary investigations on nerve fibres with the electron microscope (106, 37, 114, 119, 8, 99) have disclosed certain new structures which in many ways confirm conceptions of nerve ultrastructure derived from polarized light and X-ray diffraction studies. Although a general picture of the submicroscopic organization of nerve fibres is gradually emerging as a result of these studies, it is realized that we are dealing with an extremely complex and labile system which is difficult to analyze. By means of electron microscopy characteristic thin filaments have been observed in the axon, and indications obtained of a concentric laminated structure formed by thin membranes in the sheath. But since the preparation techniques employed involve fixation, dehydration and in many cases embedding and sectioning of the nerve fibres, the existence of these elements cannot be definitely established until the artefacts introduced by such manipulations have been adequately evaluated. The influence of these factors may be studied by systematic application of complementary preparation techniques on the same object, but a more direct approach requires the use of methods which operate as close as possible to the natural state of the nerve fibre.

This study was therefore undertaken to obtain further information on the structures described previously in fixed myelinated nerve fibres (36, 37), and is based mainly on examination of ultrathin serial frozen sections of fresh nerve. These well preserved thin sections of native, untreated nerve are prepared by an improved freezing-sectioning technique (39) and can either be frozen-dried directly or subjected to various fixation, extraction and enzymatic digestion treatments. The effects of these procedures may be followed directly by phase contrast or electron microscopy, and the method is therefore suitable for a comparative analysis of the different artefact sources. It can also be used to study the action of ultracentrifugation on the submicroscopic components of fresh myelinated fibres (39).

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[1] Professor of Biophysics, University of Caracas, Venezuela.

In a second stage of the investigation, an attempt was made to determine if the same type of structures could also be found among central nerve fibres and in the so-called "unmyelinated fibres," by extending the studies to include different kinds of central and autonomic fibres. Investigation of the smallest nerve fibres was guided by the assumption that diminishing size would gradually divest the fibres of all but their basic constituent elements, resulting in specimens thin enough for electron microscopy. This supposition proved to be correct, since extremely small fibres of submicroscopic dimensions have been found in the white matter of the spinal cord. In view of their tenuity, they afford a unique opportunity to study the simplest type of nerve fibre organization with a minimum of preparative treatment.

Thus, by applying a combination of complementary methods which stem mainly from fresh nerve and by studying different types of nerve fibres, including minute fibres which can be examined directly with the electron microscope, additional evidence is presented in this report which confirms and extends the structural details of nerve fibre organization delineated in earlier papers.

MATERIALS

Peripheral nerves (sciatic nerves and brachial plexus) from a total of 50 frogs (*Rana temporaria*), 20 albino rats and 8 cats and rabbits were examined. Motor roots, optic nerves, the sympathetic chains and the cervical, thoracic and lumbar portions of the spinal cord from each animal were included in this investigation. A few specimens from the giant fiber system of *Lumbricus* were also examined, in addition to several control samples of human peripheral and cranial nerves removed at autopsy.

The nerves are dissected out under ether narcosis observing the same precautions which are requisite at peripheral nerve operations to avoid damaging the nerve fibres or the concomitant blood vessels. In order to preserve their normal configuration and length, the nerves were ligated and affixed to small glass rods of adequate dimensions before severing off the ends with a sharp razor. The corresponding nerves of the other side, which are used for control purposes, were removed without ligating by extending carefully on a plexiglass strip inserted below the nerve before cutting off its ends.

The segments of fresh nerve are subjected immediately to the described freezing sectioning procedures or to the action of different fixation agents. Since the pieces of nerve should not have a diameter exceeding 1—2 mm, thicker nerve trunks and the segments of spinal cord have to be divided into slender columns by free-hand longitudinal sections along suitable planes.

In a number of cases the exposed intact nerve was frozen *in situ* with a jet of carbon dioxide or with liquid air, taking care to protect the whole nerve by wrapping it first in thin aluminum foil. In this way the frozen nerve can be transferred to a copper block cooled to —50° C, where suitable pieces are cut off with a cold blade and either prepared for sectioning with the freezing microtome or immersed directly in the cold fixation fluid in a still-frozen state. If properly carried out, this procedure makes it possible to examine nerve fibres which have been fixed instantaneously in a definite physiological state, before the inevitable trauma attending even the most delicate extraction can set in.

The following fixatives were applied to the thin fresh frozen sections or to the nerve segments: a) Buffered solutions (pH 7.08—7.20) of freshly prepared 1 per cent osmium tetroxide were used mainly. Special care was taken to work with neutral osmium solutions in view of the fixation artefacts produced by acid solutions (80). The thin fresh frozen sections were fixed with osmium vapours in small glass chambers through which the fixation process can be followed by phase contrast microscopy. Fixation was preferably carried out in the cold (+4° C) or at room temperature, for periods ranging between 1 hour to 24 hours, depending upon the size of the specimens. The time factor is particularly important in thin sections, because prolonged fixation with osmium will induce alterations in the fibrous structures of the axon (6). Preliminary experiments carried out in collaboration with Dr. D. Schneider to determine the rate of penetration and the effects of 1 per cent osmium tetroxide solutions on the compound spike potential of whole sciatic nerves, indicate that a marked effect sets in already after 60—80 seconds, proceeding gradually with the darkening of the nerve until all activity ceases after 6—8 minutes. b) Buffered 10 per cent formalin solutions (pH 7.00—7.2). Also formalin vapours. c) Iodine vapours applied to the thin sections for brief periods of a few minutes, according to the instructions given by Romeis (98). Iodine is an excellent fixative for electron microscopy since, like osmium, it introduces differential contrast in many of the fine cell structures. d) Uranium nitrate in formalin according to Cajal (87), and 5 per cent solutions of ammonium molybdate were also used for control purposes, in an attempt to combine fixation with selective staining of the nerve structures.

METHODS

Although many of the preparation techniques employed in this study have been described previously (35–39), a more extensive account will be presented here of the methods centering around the preparation of ultrathin serial frozen sections of fresh, untreated nerve. The principal advantage offered by thin sections of the fresh, native tissue is the possibility of direct examination with the electron microscope, or of subjecting their large exposed surfaces to the controlled action of different fixation and chemical reagents. Many of the indeterminacies inherent in working with whole specimens are thus eliminated, and the thin fresh sections have in this respect significant features in common with the tenuous layers of cells in tissue cultures. Moreover, since *the thin sections are ideal specimens for phase contrast and polarized light microscopy,* parallel studies can be carried out on the same object in order to compare the structures revealed by combined light and electron microscopy.

The degree of preservation of fine structure and integrity in the thin serial frozen sections is satisfactory in the small areas required for electron microscopy and compares favourably, or is superior in certain objects, to the structure resolution achieved by the currently employed ultrathin sectioning techniques (23, 55, 77, 99). However, structural relations involving larger areas are better preserved in embedded sections, and observations on frozen sections must therefore always be supplemented with the study of embedded material.

It is obvious that an object as complex as *nervous tissue can only be analyzed by a combination of methods,* including the dissociation and replica-adhesion techniques. Even if a sectioning technique entirely devoid of artefacts could be devised, it would be extremely time consuming, for example, to examine hundreds of ultrathin sections in order to follow the course of an individual nerve fibre over a region of a few microns only. A single suitable dissociation preparation would suffice however, as will be shown later, to display the main structural features of such a fibre, provided its dimensions are within the range accessible to the electron microscope. It was found necessary to use the variety of preparation techniques presented here, because their application ranges overlap and they generally yield complementary results. Each of these methods has proved to be of value in the investigation of nerve tissue by electron microscopy.

The following preparation methods were applied in this study:

1. *Preparation of ultrathin frozen sections from fresh nerve*

a) *Equipment.* A standard unmodified Spencer rotary microtome model No. 820 resting on a thick slab of marble was used. The microtome is fitted with an electric motor drive to deliver 30—100 revolutions per minute. A rubber transmission band connects the pulley of a small (1/12 or 1/24 hp) electric motor, which is attached to a heavy lead block and shock mounted on sponge rubber, with the microtome drive wheel (Fig. 3). A conventional freezing microtome brass stage was modified (Fig. 4) by fixing a Plexiglass disc (18 mm in diameter and 1 mm thick) to its center with 4 screws. The plastic disc has a rilled surface (Fig. 5 a) for holding the frozen specimen and serves mainly as a thermal insulator to prevent the formation of ice around the specimen, since direct contact of the tissue with the metal stage frequently resulted in disturbing ice formation when the stage came too close to the liquid reservoir of the knife. Stages of nickel-steel alloys with lower thermal expansion rates, and a modified low temperature stage of the type described by Rhodes (96) for obtaining temperatures of —100° C to —140° C by working with liquid air, were also used.

The stage is firmly clamped between two plastic blocks in the regular specimen holder of the microtome and was connected through a carefully balanced pressure hose with the carbon dioxide container (Fig. 3). The needle valves of the stage and of the special nozzle for deep freezing of the knife holder had to be carefully adjusted, so that they could be firmly closed to avoid vibrations caused by the escaping gas. Two types of knives were used routinely: thick razor blades (Schick: 38 × 8 mm) mounted in a specially constructed knife holder; and glass knives obtained by thermal fracture of 6—8 mm thick panes of plate or stained glass fastened in the special holder described by Latta and Hartmann (62). Glass knives gave excellent results and are generally superior to the steel knives, but after a short time their edge seems to be damaged by the frozen block, since they produce compression of the thinnest frozen sections.

Liquid reservoirs for collecting the sections are made from thin aluminum strips bent to form a trough (20 × 5 mm, 2 mm deep) which is fastened close to the knife edge. The trough is filled with Ringer solution, Tasaki solution (prepared according to v. Muralt, 76), or with glycerine or sucrose solutions of suitable concentrations which are frequently renewed. Frozen sections of still-frozen nerve are collected dry on detachable polished metal strips rubbed with a drop of glycerine, or on collodion-covered metal screens touching the knife edge, which can be transferred directly to copper blocks cooled to —50° C for freezing-drying in a vacuum chamber. Immediate freezing of the nerve is facilitated by inverting a metal cup over the stage when opening the valve. The sectioning process is continuously observed through a binocular microscope (25 × magnification), using a large fluorescent light placed 1 meter above the instrument to illuminate the field. The thinnest sections can only be seen properly by using a transparent plastic trough fitted with a dark field cardioid condenser.

Sectioning is carried out at room temperature or in a cold room. The microtome is carefully lubricated and run for 15 minutes before each sectioning cycle.

b) *Sectioning of fresh nerve.* Small blocks of nerve which have not come in contact with liquids and should not exceed 1–4 mm^3 in volume are oriented on a small disc of moist filter paper and frozen on to the stage of the freezing microtome (Fig. 5 b). The tissue block must be frozen solid as quickly as possible by repeated bleeding of the valve and inverting the cup over the stage. The proper degree and the rapidity of specimen freezing are decisive factors which can only be determined by continuous microscopic control of the sections at high magnifications. As a rule, the small tissue block is immediately frozen through by rapidly cooling down the stage to a temperature of —35° to —40° C as the compressed carbon dioxide expands, and any freezing artefacts which may occur are due to improper freezing or to the presence of water in the specimen. The temperature of the knife and of the collecting reservoir liquid are also important.

If the sections are to be picked up dry and in a still-frozen state, the knife must be cooled to —35° or —40° C, care being taken to prevent the characteristic folding and splintering of the brittle sections. It is easier to collect the still-frozen sections on concentrated glycerine or glycol solutions cooled to —35° C. However, for routine examinations, it was found more convenient to collect the frozen sections on Ringer or Tasaki solutions at room temperature, since the thin sections thaw and spread out instantaneously upon hitting the comparatively warm liquid surface, and thus pass through the critical temperature range unscathed. In certain cases better results are obtained by filling the collecting reservoir with a cold liquid (—1° to +4° C). Excessive hardening of the tissue due to extreme freezing, like in tissues frozen with liquid air, is not in itself a hindrance to freezing-sectioning, since excellent sections are still obtained by collecting on warm (+20° to +30° C) liquid surfaces. Undoubtedly, special conditions are operative during the brief interval when the cutting edge removes a thin layer, fractions of a micron in thickness, from the frozen block, and further progress will be made when this sectioning process is better understood.

The well frozen specimen must be free at all times from ice formation and since this depends primarily on the *adjustment of the liquid level in the collecting trough, these two factors are the most important practical details which determine the success of the method.* The meniscus of the liquid should preferably be plane or slightly convex and reach up close to the knife edge within tenths of a millimeter. If there is a slight excess the specimen picks up liquid immediately upon passing close to the knife edge, and the resulting ice formation ruins the knife edge and completely thwarts the cutting of sections which sink to the bottom in clumps. Specimens on which ice formation has occurred should be discarded, or salvaged in exceptional cases by cutting away the ice and trimming the block with a sharp razor. The well frozen specimen is advanced at 5 μ intervals until it comes in contact with the knife edge, and a series of 1 micron sections are cut with the dry knife. These first sections are discarded and the liquid reservoir is then filled, adjusting the liquid level until a suitable surface is obtained. By using collecting troughs of small dimensions adjustment of the meniscus is easy, particularly if the edges of the trough are at the same level of the knife edge, and if the aluminum trough has been sealed on tightly to the knife edge by a layer of paraffin.

A difficulty which is sometimes encountered at this stage is the erratic behaviour of the sectioning process, due either to inadequate fastening of the specimen on to the stage, which shows up in this way, or to insufficient clamping of the knife holder. Knife tilt is different for each specimen, and critical if best results are to be obtained, since it can determine regular sections or specimen fragmentation. A faint, barely audible high sound every time a section is cut indicates "good tuning" of the microtome, whereas a shrill rasp signals excessive tilt of the knife or improper fastening. Once satisfactory conditions are attained, the block is kept frozen at the suitable temperature by refreezing regularly at 1–2 minute intervals. Good 1 micron sections can already be recognized by their unmarred, transparent and fully extended serial appearance (Fig. 5 b) with diameters corresponding to the cross section of the block. Manual operation of the microtome by performing quick chopping strokes is recommended in the beginning until more practice is acquired in the use of the motor drive.

After a uniform ribbon of serial 1 micron sections has been obtained, the microtome's advance mechanism can be disengaged by setting the pointer at zero. The continuous thermal expansion of the cold stage then advances the specimen automatically at a variable rate of tenths of a micron or less, depending upon the temperature rise. The time interval between each cutting stroke must be made correspondingly shorter in this case to obtain ultrathin sections, since the temperatures necessary for freezing the specimen are much lower than those employed by Newman, Borysko and Swerdlow (77, 78). In routine sectioning, continuous refreezing of the specimen at 1–3 minute intervals and the use of a motor drive delivering a certain number of fixed revolutions per minute (30–80 r. p. m.) *produced serial sections of 0.2 to 0.05 μ thickness*. Constant section thickness can be obtained by working in a room with regulated temperature and maintaining uniform sectioning conditions. When cutting ultrathin sections of fresh nerve it is often found that the first sections disrupt and extend over the whole surface to form true monolayers; the following ones, however, are well preserved and merely compress the monolayer until a critical surface pressure is reached and the liquid must be renewed.

Good ultrathin sections flatten out immediately, are nearly invisible and adhere edgewise to form a thin uniform ribbon which advances steadily with each cutting stroke. Compression and accordion-like folding (55) occur frequently at high cutting velocities, and like other types of tissue distortion can be readily detected by continuous observation of the cutting process at low magnifications.

A series of experiments was carried out in an attempt to prevent or control the formation of ice crystals during the freezing process and still attain a suitable consistency of the tissue block for sectioning. The small pieces of fresh nerve were immersed for this purpose in glycerine-Ringer, glycol-Ringer, or sucrose solutions of different concentrations prior to freezing. At this preliminary stage of the experiments, a definite protective action of the dehydrating treatment (69, 71, 86) could be detected in fresh and fixed nerve at a concentration which was still compatible with adequate hardening of the tissue by freezing. The remarkable preservation of the whole fibre, specially in sucrose treated living nerves, even suggests the possibility of being able to restore some of the functional activity of these frozen, protected nerves after suitable thawing, like in the case of vitrified muscle fibres (72).

The main sources of trouble encountered are in approximate order of importance: (a) proper degree of tissue freezing, (b) adjustment of the liquid meniscus, (c) correct sharpness and tilt of the blade, (d) cutting rate, (e) smooth operation of the collecting mechanism, and (f) mechanical performance of the microtome. Despite these numerous factors *the method has been found to work remarkably well in routine sectioning, giving constant, reproducible results.* It demands of course more practice than ordinary sectioning procedures, and constant vigilance, but since *the simplicity of the method cuts down the usual specimen preparation time for embedding of one or more days to a few hours*, the efforts expended in preparing for sectioning are amply compensated by the rapid results obtained once the method is operating satisfactorily. Although the freezing sectioning method has already given interesting results, it can still be further improved. The use of low temperature stages operated by liquid air or liquid nitrogen seems to offer the best possibilities of achieving still better specimen preservation.

c) *Mounting the fresh frozen sections.* The thin sections floating on the surface of the liquid reservoir (Fig. 5 b) are picked up simply by lowering a collodion-covered electron microscope screen parallel to the water surface until the sections touch the flat dry film, to which they firmly adhere. The screens, in which a central slit (1.5 × 0.1 mm) can be punched out to enlarge the field of view (Fig. 5 b), are attached to small rods with an adhesive surface at one end, and deposited on the sections under microscope control. If properly carried out, this operation transfers the intact frozen sections to the plane collodion film, which provides a convenient support to the wet tissue slice over its entire surface. In order to examine the sections with the phase contrast or polarized light microscopes they are picked up on small circular glass slides or celluloid discs, and transferred to a cooled observation chamber. Frozen sections can also be picked up without thawing on deep-frozen electron microscope screens covered with collodion or metal films; and on undercooled glycerine or glycol.

d) *Freezing-drying of thin frozen sections of fresh nerve* (39). If the fresh frozen sections of nerve, which have been picked up from the Ringer solution are simply air-dried, collapsing of the myelin sheath and other serious distortions due to the surface forces ensue. Many of these artefacts can be avoided if the sections are frozen-dried (39), or subjected to the critical point drying method described by Anderson (2). Anderson's method requires dehydration of the specimen in alcohol and is therefore unsuitable for fresh nerve sections, unless glycerine, sucrose or some similar dehydrating agent can be substituted for the alcohol. A modified freezing-drying procedure which was described by Wyckoff (128) for electron microscope preparations has been used instead (39), and helps eliminate many of the drying artefacts in thin fresh frozen sections. At the same time important details of the three-dimensional structure can be preserved in certain regions of the object.

For this purpose, the still-frozen sections mounted on the cooled grids are placed immediately on a block of copper cooled with dry ice to —45° C, and the block is quickly transferred to an insulating plastic base in a vacuum chamber, which is promptly evacuated. Condensation of air moisture on the sections cannot be prevented despite rapid transfer, and this always introduces a certain amount of specimen distortion. The frozen water in the sections sublimes directly in the high vacuum under unusually favourable conditions, since the sections are ordinarily only fractions of a micron in thickness and present a large exposed surface. The solid-vapour boundary moving through the specimen during sublimation of the frozen matrix has therefore less possibilities of disrupting the nerve structures than in the commonly used freezing-drying methods, where the specimens are much thicker. After reaching a high vacuum which is maintained for 1—2 hours to ensure complete dehydration, the sections are shadowed by oblique evaporation of platinum/palladium at a 10—30° angle, followed by vertical deposition of a reinforcing aluminum or silicon monoxide film 50—100 Å thick, without breaking the vacuum. Deposition of an additional film of these dimensions on the free surface of a thin section (1000—3000 Å in thickness), which is already firmly anchored to the collodion film, provides adequate support when it is removed from the vacuum.

Despite these precautions the frozen-dried thin sections often show considerable distortion in certain areas on which probably ice crystals had formed. But the most serious drawback of the frozen-dried fresh sections was the presence of numerous crystalline particles of all dimensions, which covered the entire surface and obscured much of the fine detail. Only the external collagen fibrous layers of the sheath were well preserved (Fig. 18), and the fibrous structures of the axon. Better results were obtained with frozen sections which had been floating on undercooled glycerine (—40° C) and were transferred to the vacuum chamber, where they could be subjected to the freezing-drying procedure and the metal films evaporated directly on to the sections because of the glycerine's low vapor pressure (49). The frozen-dried fresh sections have not as yet shown the expected structure resolution, mainly due to the contaminating crystalline particles derived from the precipitated salts, and because the native, unfixed sections are unstable and volatilize readily under the electron beam. However, the few successful preparates display a striking three dimensional quality in stereoscopic pictures and suggest that the method can eventually be improved. *Freezing-drying of fixed ultrathin frozen sections by this method gives excellent results*, provided the sections are almost completely drained of their water content before freezing.

2. *Fixation of fresh nerve sections*

Frozen sections of fresh nerve one micron or less in thickness, which have already been mounted on the collodion supporting films, are very well suited for fixation with different reagents, particularly since the results can be followed at appropriate intervals with the phase microscope and the electron microscope. For this purpose they can be fixed like tissue culture cells with osmium, iodine or formalin vapours, by placing the inverted grids in a drop of Ringer, (or dry while still frozen), over a drop of reagent in a sealed concave slide. Floating of the sections directly on the fixative can also be tried. Considering the tenuity of the nerve sections, fixation times must be made correspondingly shorter. This applies particularly to fixation with osmium tetroxide which induces marked alterations after protracted action. *A comparative study of fresh frozen sections from the same nerve segment, which were subjected either to freezing-drying or to parallel osmium fixation, shows that the axon and sheath structures revealed in both are nearly identical,* but better preservation and contrast was achieved in the osmium fixed sections (Figs. 23, 24). *For routine examinations osmium fixation of the thin fresh frozen sections would therefore appear to be the method of choice.*

3. *Complementary procedures for fresh nerve sections*

Thin frozen sections of fresh nerve can be further subjected to a wide variety of different treatments:

(a) *Enzymatic digestion.* The fresh frozen sections which have been picked up on collodion-covered stainless steel grids or on glass slips covered with a thin collodion film, are transferred to a hanging drop of the buffered solutions of the enzyme under investigation (trypsin, pepsin, ribonuclease, lipases, etc.). The grids are attached to a thin cover slip which is sealed on to a glass ring cemented on a glass slide maintained at constant temperature. The structural modifications induced by digestion are readily apparent in the thin, exposed section and can be followed step by step under the phase microscope. Since structural relationships are generally well preserved in the section due to the supporting action of the collodion film, the process can be stopped at suitable intervals by fixing the sections. In this way sequential studies on the effects of enzymatic digestion can be carried out under electron microscope control.

(b) *Extraction experiments.* In order to extract fresh nerve sections with alcohol, acetone or any of the other lipid solvents, the sections must be mounted on grids covered with thin aluminum or silicon monoxide films. Since the action of the solvent is often extremely rapid, it is best to place small drops on the wet sections and to check the results with the phase microscope until visible changes in the sheath have been produced. If the drop is allowed to evaporate on the same grid, the dry dissolved residue of the small quantity of material contained in the thin section can also be examined. A similar procedure can be followed when testing the effects of chemical reagents on the nerve sections.

(c) *Microincineration experiments.* Ultrathin frozen sections which have been subjected to the modified freezing-drying procedure are especially suitable for studies on the localization of inorganic tissue constituents by means of microincineration. For this purpose the fresh sections are mounted on thin (100 Å) aluminum or silicon monoxide films, where they can either be incinerated within the electron microscope by electron-induced microincineration, or in an electric oven as described previously (35, 39). Loss of many of the volatile substances and migration of the mineral residue in the specimen can be prevented by depositing a silicon monoxide or aluminum supporting film on top of the specimen before incineration. This "Replica-incineration" procedure (35) preserves a cast of the original specimen in addition to the mineral residue which is enclosed between the two metal films. Electron diffraction patterns (35, 39) can also be recorded from certain types of mineral residues. Several microincineration experiments have been carried out on a number of fresh frozen nerve sections, but the microcrystalline salt contamination makes it difficult to draw any conclusions on the natural distribution of inorganic constituents in the fibre.

4. *Preparation of ultrathin frozen sections of fixed, unembedded whole nerve*

Ultrathin frozen sections of unembedded nerve fibres fixed with neutral osmium tetroxide and formalin solutions were used extensively throughout this study. As has been pointed out previously (37, 39), osmium tetroxide is the most suitable fixative for freezing-sectioning, because it preserves and stabilizes the fine structure of nerve very well, and imparts a firm consistency and rigidity to the tissue. Many of the finest structures in the myelin sheath have only been found in osmium fixed whole nerve (37, 119), but the presence of osmium precipitation products in the thin sections (38) must constantly be kept in mind. Sectioning of osmium fixed nerve is easily accomplished (Fig. 4 a) and the serial sections can be further subjected to the modified freezing-drying procedure. Formalin fixation is inadequate for freezing-sectioning of nerve, since the lipids have a tendency to migrate and contaminate the other components of the section, giving rise to spurious structures.

5. *Preparation of ultrathin sections of embedded material*

Ultrathin sections of osmium fixed nerves embedded in methyl-methacrylate according to the method of Newman, Borysko and Swerdlow (77, 78) were also examined for control purposes. The only modification introduced involved the use of glycerine instead of alcohol for dehydration of the nerve. The fixed nerves were run up through graded series of glycerine solutions until they could be transferred to concentrated glycerine (98—99 per cent) where they remained for several hours. After wiping off the excess glycerine the small nerve pieces were placed in several changes of monomer before following the ordinary embedding procedure. The glycerine dehydrated nerves are sometimes hard and brittle, but give satisfactory sections with the glass knife (62), and show good preservation of the myelin sheath structures. Embedding in 10 per cent and 20 per cent gelatine solutions according to the method of Heringa and ten Berge, and in dilute egg albumen solutions hardened subsequently in formalin, proved to be valuable in connection with the preservation of axon structures. Gelatine embedding is particularly useful in defining the minute topography of large areas which the freezing sectioning technique cannot preserve adequately.

6. *Isolation of single nerve elements by microdissection and dissociation*

The isolation procedures contributed an interesting set of findings in the special case of the white matter of the spinal cord, where a large number of extremely fine nerve fibres suitable for electron microscopy could be made available for examination by the simple expedient of careful dissociation. If the limitations of the method are kept in mind, it is still one of the most valuable and straightforward preparation techniques. The variants employed can be divided into:

a) *Microdissection of fresh nerve trunks.* Spinal cord segments of the frog or the rat which have been carefully removed and divided into thin columns, can be easily separated into smaller individual bundles by teasing on a cold plate (+4° C) with sharp needles under the binocular microscope. The absence of connective tissue sheaths facilitates the unravelling process once the stickiness of the fibres is overcome. Without damaging the larger fibres, the smallest fibres can be pried loose by merely widening the gap between larger bundles. Once this stage has been reached, a small quantity of osmium tetroxide solution is added and the selected small fibres are sucked up with a micropipette after waiting for several minutes. Examination with the electron microscope can proceed after transfer to collodion grids, simple air-drying or freezing-drying and shadowing.

b) The second method involves the use of thin (1—10 μ) fresh longitudinal frozen sections which can be dissected and the isolated elements fixed in the same manner, but it affords the additional advantage of a more precise localization. Frozen longitudinal sections not thicker than 10—20 microns can also be fixed in osmium tetroxide solutions for short periods and then easily dissociated into the finest nerve fibrils. Localization of the isolated structures is facilitated by the use of thin frozen sections which contain a limited number of elements only.

c) Nerve tissue fixed with osmium solutions has a well known tendency to dissociate readily into its finest components (98), and this property can be put to use in a more controlled fashion by working with thin frozen sections. Thin (0.2—5 μ) longitudinal and transverse frozen sections of osmium fixed whole nerve, enclosed between a glass slide and cover slip, can be carefully dissociated under light microscope control by gentle tapping. Sonic fragmentation combined with separation of the different structures through centrifugation is also particularly successful when carried out on ultrathin osmium fixed sections. Finally, an extreme degree of specimen fragmentation can be produced by changing the angle of the knife during freezing-sectioning, so that the knife edge merely scrapes ultrathin layers off the fixed, frozen nerve.

7. *Replica-adhesion preparations*

Thin and ultrathin fresh frozen sections which are deposited on glass slides and either frozen-dried or fixed with osmium vapours, can be subjected to the replica-adhesion techniques by covering with a 5 per cent gelatine solution or with 1 per cent collodion in amylacetate. The resulting thick film is stripped off,carrying with it a tenuous surface layer of the section, the bulk of which sticks firmly to the slide. By carefully dissolving the gelatine or collodion films coherent sections thinner than 0.1 μ can be obtained.

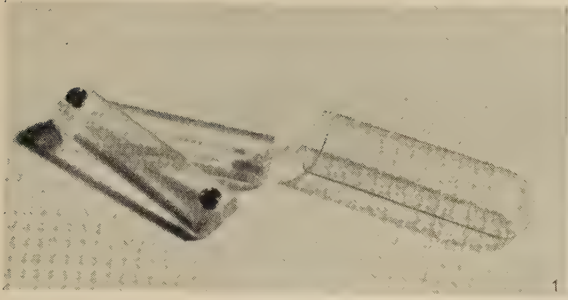
8. *Ultracentrifugation experiments*

When subjecting fresh nerve to ultracentrifugation it is often difficult to remove the tissue, which has been tightly wedged into the container by the centrifugal force, without distorting it. In order to avoid this, a special plexiglass chamber was used (Fig. 1) which consists of the two halves of a solid plastic cylinder carefully ground and fitted together by means of two screws. A central groove is made in each piece, adapted to the dimensions of the nerve to be centrifuged. The fresh nerve is placed into the moistened groove immediately after removal and a close fit secured without compressing it. The other half is then screwed on and the chamber inserted into the regular plastic tubes of the ultracentrifuge. The nerves are centrifuged for 30 minutes to 2 hours in an electric ultracentrifuge at a speed generating a force of 20 000 times gravity, or in an air-driven ultracentrifuge generating forces up to 100 000 g. After completion of the centrifugation observing the usual precautions, the chamber is carefully opened and the piece containing the nerve is immersed directly in the fixative, or fastened on to the microtome stage for freezing-sectioning of the nerve.

9. *Electron diffraction experiments*

For the special purposes of this investigation it was necessary to record electron diffraction patterns from lamellar structures which are only a few microns long. Several methods (17, 4, 29) have been described for observing electron diffraction patterns from restricted areas of a specimen. By using a small condenser aperture in an RCA EMU electron microscope, it is also possible to record diffraction patterns from limited areas of a specimen according to Picard and Reisner (85), and Boadway (15). But this can only be done by breaking the vacuum and removing the pole pieces, which would prove impractical for routine work involving a large number of observations. A more suitable method, by means of which the diffraction pattern can be recorded directly from the part of the specimen being examined without discontinuing the observations, was originally proposed by Boersch and applied in the Philips electron microscope. This is achieved primarily by using an intermediate lens between objective and projector so that the focal plane of the objective lens on the image side is displayed in the object plane of the projector lens and therefore projected onto the fluorescent screen.

While using a fine condenser aperture of 200 μ according to Boadway (15), in combination with a removable intermediate lens (53) fitted with special apertures in an RCA EMU electron microscope, it was noticed that the electron diffraction patterns from small areas of the specimen under examination could be observed on the final viewing screen directly, by turning down the magnification controls to the lowest setting (39). In this case the diffraction spectrum of the specimen structure, which appears in the focal plane of the objective, was being projected on to the final viewing screen because the excitation of the intermediate lens was just adequate to accomplish this when the accelerating voltage was 40 kV instead of the usual 50 kV. Subsequently, the current in the intermediate lens was slightly increased when using 50 kV to obtain the same result, by setting the magnification of the primary image to zero.



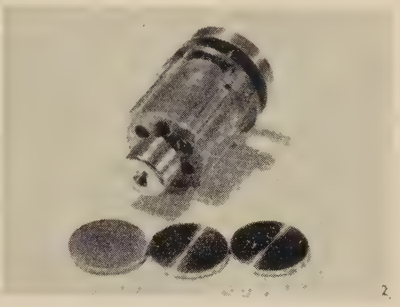


Fig. 1. Opened plexiglass chamber with central groove for ultracentrifugation of fresh nerve. 1:1.5.
Fig. 2. Device for holding the fine condenser aperture according to Boadway, together with a set of apertures for the intermediate lens used in electron diffraction work.

a) *Arrangement for recording electron diffraction patterns of restricted specimen areas.* With the present arrangement it is possible to record successively electron diffraction patterns and electron micrographs from restricted areas (10—50 μ) of the same object in the specimen holder of an RCA EMU electron microscope, without breaking the vacuum.

The regular condenser aperture of an RCA EMU electron microscope with a biased gun and an accelerating potential of 50 kV is replaced by a non-contaminating platinum (25 μ thick) condenser aperture of 200 μ, inserted in the special device (Fig. 2) described by Boadway (15). (A leaf spring placed between the outer piece and the barrel facilitates the centering procedure.) The current in the intermediate lens was slightly increased by connecting a 10 000-ohm rheostat in series with the 1500-ohm shunt resistor, inserting a switch in parallel with the rheostat to restore original conditions when desired. Intermediate lens apertures of different sizes, ranging between diameters of 1 mm and 0.2 mm, were introduced to restrict the fields from which diffraction patterns are recorded.

In operation, a suitable area of the specimen in the normal object position is centered on the viewing screen after taking the corresponding electron micrograph, and the electron beam is focused to an area of an approximate cross section of 20—50 μ on the specimen film. It is now merely necessary to turn down the magnification controls (projector and intermediate lens) to the lowest setting, and to adjust the current in the intermediate lens with the 10 000-ohm rheostat, in order to obtain a brilliant electron diffraction pattern of the specimen on the viewing screen.

The fine condenser aperture must be properly aligned, of course, following the procedure described by Boadway (15). A better definition of the pattern is often obtained by turning the projector lens on and off repeatedly, in order to obliterate the previous magnetization history of the pole pieces. The objective lens must also be used without an aperture. *Only short exposure times of the order of 1/10 to 1/2 second are needed to record the patterns,* and this is of particular importance when studying volatile substances like lipids and fatty acids (124). The relatively high beam intensity also makes it necessary to work rapidly, since contamination of the specimen with a masking carbonaceous deposit is only a matter of seconds. Once the pattern has been recorded, the controls are switched back to restore the electron microscope image, and the preparation is scanned for crystalline structures. These can often be readily recognized by their characteristic "reflection images" (19, 51) and "extinction contour" patterns (18, 50).

Calibration is carried out by recording electron diffraction patterns of an evaporated gold film under standard conditions. However, for more critical work and to obtain resolution of longer spacings, the arrangement described by Picard and Reisner (85) is used. For this purpose the specimen is left in position and the objective and projector pole pieces are removed. After minor adjustments in the microscope alignment and insertion of the central beam stop of Picard and Reisner (85), diffraction patterns can be taken at different projector settings. In suitable cases it might be possible to record spacings up to 10 Å.

The described arrangement is therefore suitable for investigation of certain organic compounds because a direct correlation between electron microscope image and electron diffraction pattern is possible, and only short exposure times are required. Moreover, the accuracy achieved is satisfactory for most purposes, and in certain cases the adapted microscope can be used as a high resolution electron diffraction camera (85).

Throughout this work an RCA EMU electron microscope with an accelerating voltage of 50 kV, a biased gun and a removable intermediate lens was used. It was also equipped with a fine condenser aperture of 200 μ, a compensated objective lens and a telescopic image viewing device.

RESULTS

1. *Peripheral myelinated nerve fibres*

Only the structures in the internodes will be described here, since it has not been possible to obtain an adequate number of satisfactory sections through the nodes. Moreover, the basic structures of the nerve fibre are represented in the internode portion.

Thin transverse sections of fresh rat or frog sciatic whole nerve picked up on Ringer or Tasaki solution (Fig. 6), show well preserved bundles of nerve fibres enclosed in the connective tissue compartments of the endoneurium. The individual fibres appear under phase contrast as glistening, perfectly homogeneous rings (Fig. 7 a) with an optically empty axon. Under the polarized light microscope the frozen sections exhibit the same properties as isolated fresh fibres, and the slightest damage is immediately apparent in the sheath. A combination of phase contrast with polarized light (7) is also useful for checking on the preservation of the sections. If such a 1–0.5 μ thick section is examined with the electron microscope after freezing-drying or fixation with osmium vapours (Fig. 7 b), its higher resolving power discloses the main structures: a dense myelin sheath from which layered membranes flow out at the edges, enveloped by external fibres with a typical collagen periodicity, and containing a dense net of delicate filaments strung across the axon. The thin nodose filaments 100–200 Å in diameter are associated with small vesicular bodies (0.1–0.3 μ) and dark granules. Fine structures are clearly discernible in such a comparatively thick section because the many empty spaces are unobstructed by an embedding medium, and the electron microscope's considerable depth of focus permits a three-dimensional view. This represents a typical advantage of frozen sections which is of special value in the study of axon filaments.

1. *Structure of the myelin sheath*

a) *The myelin lamellae.*

Previous electron microscope investigations by Sjöstrand (118), Schmitt (108) and the present author demonstrated thin layered membranes in dissociation preparations of fixed nerve. These lamellar layers could be localized to the myelin sheath in frozen sections of osmium fixed nerve (36, 37). In such thin sections the lamellae appear as piles of thin, uniform, flexible unit membranes. The layers have been found in all types of peripheral myelinated nerves examined. The thinnest unit lamellae in the frog have a thickness of 70 Å, while thicker ones of 80–90 Å are found in rat, cat (Fig. 8), rabbit and human nerves, with an average thickness of 80 Å. (Addition of spherical Dow Latex particles with a known diameter increases the accuracy of the thickness determinations.) A granular surface structure consisting of 20–50 Å particles is a distinguishing feature in shadowed preparations. The lamellae appear orderly stratified in plane formations with minor creases, and have a tendency to disintegrate into small granules. The layered membranes found at the edges of air-dried, frozen-dried or fixed fresh sections have rounded, irregular margins but are otherwise identical with the fixed myelin lamellae. Small myelin droplets released from teased fresh fibres show a similar type of multiple layers with filiform extensions.

Digestion experiments: Whole nerves digested with trypsin (37) show myelin lamellae which disintegrate into rod shaped granules 50–100 Å high and 20–40 Å wide without indications of a separate thin supporting membrane. Repetition of the experiments on different nerve types has confirmed these findings. Upon digesting fresh frozen sections with trypsin a characteristic swelling of the sheath is observed, and electron microscope examination reveals that the whole sheath has separated into globular formations or layers. Thick fibrous residues are seen between the granules of the disintegrating membranes.

Extraction experiments: When whole nerve is extracted with alcohol-ether and subsequently osmium fixed (37), thin leaflets are seen in longitudinal frozen sections. However, if fresh nerve sections are treated with alcohol the myelin flows out and forms profuse, vacuolated microdroplets leaving a distorted, fenestrated sheath. Chloroform extraction is more mild, but these observations emphasize how drastic the effects of alcohol extraction are, particularly on thin, unsupported sections.

Preliminary microincineration experiments carried out on fresh frozen sections with numerous myelin layers show that most of the membranes disappear, leaving only faint droplets and indications of dispersed layers of dense particles in some areas. The violent bubbling of the membranes during incineration produces serious distortions.

Ultracentrifugation experiments: Centrifugation of nerve tissue was introduced by Arndt (5), Ingvar (59), and ultracentrifugation of spinal ganglia by Muliyil (75), Beams and Kirschenblit (9) in order to bring about displacement and separation of cell constituents. The application of ultracentrifugation of fresh whole nerve in connection with electron microscope studies (39) was based on the assumption, that displacement or warping of submicroscopic components within the fibre would furnish indirect proof of their existence as preformed elements, and represent at the same time a reproducible form of microdissection at this level.

If whole fresh rat or rabbit nerves are ultracentrifuged in the special plexiglass chambers (Fig. 1) and fixed immediately afterwards while still lodged in the central groove, or subjected to freezing-sectioning, the nerve fibres present a characteristic picture. The myelin sheath has been partially or completely detached from the external fibrous sheath walls, forming folded, serpentine figures (Figs. 14 a, b). It does not coalesce to become a single droplet under the effect of ultracentrifugation, nor is the thickness of the myelin layers appreciably increased. These figures are clearly different from the typical myelin figures seen in damaged fibres. The outlines of the myelin layers are not obliterated despite the folding, but incisures can no longer be discerned.

Electron microscope examination of thin, frozen, osmium fixed sections of the ultracentrifuged fibres (Fig. 15) reveals only heavily folded and creased myelin membranes. Instead of the plane, neatly stratified layers encountered in normal preparations one finds this complex folding and multiple creasing of the otherwise unaltered lamellae. The unit lamellae have the same thickness as in normal nerves and are still able to separate from the stratified formations. We are evidently dealing here with *a warping of the laminated* *sheath structure produced by ultracentrifugation.* If the lamellar membranes revealed in the sheath by electron microscopy were the result of a spurious lamination produced by fixation or desiccation, the lamination observed in ultracentrifuged fibres would probably exhibit the same uniform, stereotype character. Instead, this characteristic wrinkling of the layered structures is observed, which can be explained by assuming that the membranes were already present as natural constituents of the myelin sheath when the nerve fibre was subjected to ultracentrifugation.

b) *Crystalline structures resembling myelin lamellae in osmium fixed preparations.*

It was pointed out in a previous communication (38), that certain thin lamellar structures exhibiting typical crystal interference phenomena are found in large numbers among the real lamellar membranes of the sheath in osmium fixed preparations. These crystalline lamellae, which have been tentatively designated "osmium lamellae," are thin (50–60 Å) smooth, plane flakes with angular edges (Fig. 9), occurring in piles of various thickness up to 30–40 layers (Fig. 12). In unshadowed preparations they are often difficult to distinguish from the myelin lamellae, but larger specimens demonstrate crystal interference phenomena like dark wavy lines corresponding to extinction contours (Fig. 9), or "reflection images" constituted by diffuse light patches observed close to the lamellae. The distinguishing granular surface structure of the myelin lamellae (Fig. 8) contrasts with the perfectly smooth surfaces of the osmium lamellae (Fig. 9) in shadowed preparations.

With the described arrangement (38) for recording electron diffraction patterns from restricted areas of a specimen together with the corresponding electron microscope image, it could be demonstrated that the thin osmium lamellae give typical cross-grating electron diffraction patterns (Figs. 10, 11). Cross-grating diffraction patterns with multiple spots were obtained from thicker piles of osmium lamellae, and the thickest ones (Fig. 12) give typical Kikuchi line patterns (Fig. 13). A possible contamination with extraneous crystalline material could be ruled out, nor did the collodion supporting films used exhibit a polycrystalline or single crystal structure (41).

The intimate association of osmium lamellae with myelin lamellae in osmium fixed preparations indicated that the crystalline lamellae might be osmium products deposited in the sheath. However, the osmium deposits ordinarily found here are small, dense granules which rarely exhibit a distinct lamellar structure. The striking resemblance of the crystalline structures with the myelin lamellae suggested that they might be osmium-lipid compounds. In order to test this possibility, dried alcohol-ether extracts of whole nerve were treated with 1 per cent osmium tetroxide solutions for periods ranging between 6 and 48 hours. Upon examining the suitably dispersed black reaction product with the electron microscope, typical crystalline lamellae were found which give the same type of cross-grating diffraction patterns as the osmium lamellae of fixed preparations. Associated fine crystalline granules and amorphous droplets resembled the ordinary osmium fixation products and failed to give cross-grating patterns. Interpretation of the recorded electron diffraction patterns is still in a preliminary stage, and at present it is not possible to determine if the crystalline lamellae represent ordinary osmium reaction products or osmium-lipid compounds. Their typical lamellar structure and close association with the lamellar structures of the myelin sheath would favour the latter possibility.

c) *The concentric laminated structure.*

With improved sectioning techniques, the concentric laminated structure of the myelin sheath (37) could be demonstrated with greater clarity in thin osmium fixed frozen sections of all nerve types examined. It consists of a system of parallel dark lines (approximately 20–30 Å wide) separated by lighter spaces, with a regular period of 60–80 Å. The largest periods (80–90 Å) are found in cat and rat nerves, while the smallest spacings of 60–70 Å were recorded in the sheaths of frog sciatics (Fig. 16). The dark lines are ordinarily uniform, but in certain preparations discontinuous segmentation is seen with concomitant irregularities in the periodicity. The concentric laminated structure can be followed over large segments of the sheath, particularly in smaller fibres with thin sheaths. In suitable preparations, direct transitions can be seen where the laminated structure separates into thin lamellar ribbons. The system of concentric striations is sometimes associated with dense irregular particles which show a concentric arrangement parallelling the laminated structure, and might correspond to oriented osmium reaction products (102, 103). The concentric grating structure (37) described previously as an extremely regular system of lines associated with the laminated structure was mainly found in areas containing a large number of oriented osmium deposits. It might bear a certain relation to the crystalline osmium lamellae which would also account for its precise configuration. The concentric laminated structure has only been seen in osmium fixed myelin sheaths, since ultrathin sections of fresh nerve are not stable enough to exhibit this degree of fine structure.

d) *The neurilemma and the Schwann cells.*

The granular neurilemma membrane surrounding the myelin sheath shows the same structural features and average thickness of 200–300 Å in all nerves examined. Longitudinal bundles with characteristic collagen periodicity are regularly attached to the neurilemma and appear particularly well preserved in frozen-dried fresh sections (Fig. 18). Smooth fibrils, 100–200 Å in diameter and bound together in compact longitudinal strands (Fig. 17) are also found attached to this membrane, and may be related to the elastic fibres of the sheath (37). The continuity of the neurilemma membrane with the protoplasm of Schwann cells can be demonstrated in suitable sections. Characteristic dense oval bodies 0.3–1 μ in diameter (Figs. 19, 20) and consisting of a granular material which can be extracted with acetone or alcohol-ether, are found in large numbers in the vicinity of the Schwann cells. In replica-adhesion preparations (Fig. 20) indications of a surface membrane can be seen. Judging from their size and appearance under the light microscope they probably correspond to the Reich granula (93, 125). The Schwann cell nucleus contains several dense round bodies embedded in a compact granular material, and is surrounded by a large number of corpuscles of different sizes.

e) *The incisures of the sheath.*

Oblique clefts in the sheath corresponding to the Schmidt-Lantermann incisures can be clearly seen in longitudinal frozen sections of fresh nerve fibres (Fig. 21 a, b). Examination of these incisures with the electron microscope after freezing-drying or osmium fixation reveals distinct oblique gaps (Fig. 22) in the sheath (approximately 0.5–1 μ wide after drying) which are either empty or filled with a loose granular material. In some preparates contamination with axon filaments or collagen fibrils is also observed. The partition extends from the axon to the external sheath layers where it is reinforced by the neurilemma membrane and its fibre bundles. According to these findings the clefts would seem to represent natural communication channels of the axon with the exterior of the fibre, and it might even be possible to insert micro-electrodes through these oblique gaps into the axon at the internodes.

2. *Structure of the axon*

Satisfactory thin (0.2–1 μ) longitudinal frozen sections of fresh nerve fibres collected on Ringer or Tasaki solutions show no visible structure in the perfectly clear axon (Fig. 23 a), when examined with the phase microscope. However, upon being subjected to the prolonged action of osmium vapours faint indications of a diffuse fibrillar structure can be made out. This leads eventually to the formation of long slender fibres resembling neurofibrils, particularly in the case of thicker sections. Electron microscope examination of these sections after freezing-drying or simple air-drying discloses an evenly distributed network of predominantly longitudinally oriented thin smooth filaments with a diameter of 100–200 Å sprinkled with precipitated salt particles.

a) *The axon filaments.*

In a series of experiments carried out in collaboration with Dr. G. Svaetichin, the axon material of the giant fibres of *Lumbricus* was sucked up with a micropipette and deposited directly on the collodion membranes of the screens. Here too, the axon material, which was uncontaminated with sheath components and simply air-dried, shows contorted filaments 100–200 Å in diameter, which resemble the fibrillar structures described by Richards, Steinbach and Anderson (97) in extruded squid axoplasm.

Better preservation is obtained through osmium fixation of the fresh 1 μ thick sections where the course of the filaments is discernible over large areas (Fig. 23 b). In contrast to the thin sections of embedded nerve where only short segments of the filaments are seen, the individual filaments can be followed over stretches of 50 μ or more in these thick unembedded sections (Fig. 24 b). Interlacing of the oblique and longitudinally oriented filaments is probably due to rearrangements introduced during the preparation treatment, because many sections show a distinct parallel disposition (Fig. 26) of the filaments. The individual filaments have diameters of 100–200 Å with axial discontinuities spaced at regular intervals of 200–300 Å. This nodose appearance is seen only in fixed material, since dried native filaments are generally smooth. In sections which have not been fixed too long, there is an even distribution of the filaments over the central part of the axon, and only the regions adjacent to the sheath walls show a typical concentration of filaments. Protracted fixation with osmium leads to the formation of longitudinal filament bundles corresponding to the neurofibril type of structures seen with phase contrast. Even longer fixation times result in condensation of the entire filament material into compact membranes. Fixation of the fresh sections with formalin often yields dense-edged tubular structures in certain areas which are contaminated with sheath membrane material.

Ultracentrifugation experiments on whole fresh nerve produce characteristic stratified "meniscus" formations within the axon of the fibres, and upon electron microscope examination a *typical displacement and amassment of the filaments* is seen in these regions. Most of the filaments retain their individual appearance, but others are compactly agglutinated into contorted strands. These results could be interpreted as indicating that the filaments are preformed natural elements of the axon which have been displaced by ultracentrifugation. The axon filaments are resistant to extraction with alcohol, acetone and chloroform, but digestion with pepsin and trypsin destroys them. Microincineration experiments reveal a comparatively large amount of mineral residue in the axon, but this may be due to the usual contamination with salts. In a few cases fine linear rows of minute granules were found, suggesting the residual ash of axon filaments.

A dense fibrous net resembling the axolemma membrane (37) of osmium fixed whole nerve was not encountered in fresh frozen sections, although a greater concentration of filaments lining the internal sheath wall and associated with sheath membranes is regularly seen.

b) *The opaque bodies in the axon.*

Rod-shaped or round dense bodies have been described in the axon by different investigators (37, 114, 8, 99), and occur regularly in every section. The opaque bodies of different sizes and shapes are embedded among the fibrils and many of them can be easily seen with the light microscope. The smaller ones (Fig. 25) are roughly spherical with a dense nucleus and occasional indications of a membrane. Extraction with alcohol-ether or acetone does not affect them appreciably. In frozen-dried or osmium fixed fresh frozen sections numerous small round, transparent vesicles or bladders are seen (Figs. 23, 24, 25) with diameters ranging between 0.2 and 1 μ. These globular bodies occur in connected rows or conglomerates and seem to represent collapsed vesicles in view of their thin wrinkled membranes. The bladders are similar to the sheath membranes, but the latter contaminate the axon only in the form of irregular layered patches which are different from these globular formations. In fresh frozen sections a certain contamination with sheath components is inevitable, but in preparations where the vesicular bodies are particularly numerous only limited admixture of myelin membranes is noted in the axon. They are removed by alcohol extraction and seem to represent lipid formations of some kind. Schmitt (105) has obtained lipids from extruded squid axoplasm, and it is possible that these vesicular bodies are either lipid structures in the axon or merely contaminating sheath components.

2. *The small nerve fibres in the white matter of the spinal cord*

The search for nerve fibres of small calibre, which might display their entire structural pattern in a compact form suitable for electron microscopy, was particularly successful among the fibre tracts of the spinal cord. Myelinated nerve fibres of 2 μ diameter or less are found in large numbers in the white matter of the spinal cord, where they are associated with even finer fibres which approach the limits of visibility. Cervical and thoracic segments of the frog or rat spinal cords removed with intact sheaths can be easily halved by a longitudinal section passing through the anterior median fissure, and each half further divided into long columns by sectioning along the postero-lateral sulcus parallel to the median fissure. In this way two slender cords consisting almost entirely of white matter (lateral funiculus) are obtained, which can be handled like nerves, although they are bounded on one side only by sheaths. By placing the cords on a cool plate (+4° C), free-hand wedge shaped longitudinal sections only 30–80 μ thick in certain areas can be prepared with a sharp razor moistened in Ringer solution. These fresh sections are suitable for phase contrast microscopy when transferred to a drop of Tasaki solution, or for fixation with osmic acid.

Examination with the phase contrast microscope reveals 4 main types of nerve fibres packed closely together in bundles:

(a) ordinary cylindrical myelinated fibres, 5—10 μ in diameter, with thin, straight sheaths, distinct incisures and numerous collaterals (Fig. 29 a).
(b) Myelinated fibres of smaller calibre (2 μ or less) with typical bulb shaped, symmetrical enlargements resembling tiny micropipettes (Fig. 29 b, c).
(c) Extremely fine fibrils attached to the myelinated fibres with bulbous swellings, or lying free at the edge of the section (Fig. 29 d, e). These fibrils have characteristic fusiform enlargements, giving them the beaded or varicose appearance by which nerve fibres are commonly recognized among the connective tissue fibrils in the central nervous system or in the autonomic system (122).
(d) Sharply outlined straight, dense fibres of varying diameters (0.2—5 μ), which seem to have no sheaths (Fig. 29 f, g), and would therefore correspond to the unmyelinated nerve fibres (20) and naked axons (91) of the spinal cord.

Since these preparations are obtained with a minimum of manipulations, and the observations were made on fresh, unstained fibres in the intact sections by phase contrast microscopy, the fusiform enlargements cannot be regarded as artefacts, like has often been suggested (122). Separation of the fibres is readily accomplished under a binocular microscope with sharp needles and micro-spatulas of glass, using the procedures described for isolation of single peripheral nerve fibres (76). The absence of connective tissue sheaths facilitates their separation, and microdissection experiments such as pulling off the myelin sheath from the axon can also be carried out with ease on the larger fibres. Localization of the different fibres within the spinal cord is not difficult, and contamination with the gray matter can be avoided if the sections are confined to areas adjacent to the membranes of the cord.

The observations recorded here were primarily made on nerve fibres of these longitudinal segments of the lateral funiculus of the frog cervical cord, which are either dissected fresh for the purpose of isolating and fixing the single fibres, or fixed in toto and subsequently dissociated. One half of each lateral funiculus used for the dissociation preparations was subjected to the freezing-sectioning methods, or to conventional ultrathin sectioning techniques after methacrylate embedding, in order to determine the localization of the different fibre types with greater accuracy. The absence of a detectable myelin sheath in the fine fibres with fusiform enlargements and in the dense, straight fibres would justify their classification among the so-called "unmyelinated" nerve fibres. However, it will be shown presently that these fibres not only possess a well defined sheath which exhibits the same fine structure as the myelin sheath of the ordinary myelinated nerve fibres, but that their very tenuity renders them unusually favourable objects for electron microscopy. A large proportion of the fibres are even beyond the limit of visibility with the light microscope, and these fibres of submicroscopic dimensions display in their simplest form the basic structural elements of myelinated nerve fibres. The different kinds of nerve fibres will be described separately, taking into account that the unmyelinated nerve fibres of the spinal cord and of the autonomic system show certain structural features which warrant their inclusion in a special group.

1. *The small myelinated nerve fibres with fusiform swellings*

Larger myelinated nerve fibres of the white matter of the spinal cord, with a diameter of 5–10 μ, are regular thin-walled cylinders, but when they have collaterals of 2 μ diameter or less (Fig. 29 a), these generally show characteristic bulbous swellings. The enlargements are seen only in the smaller fibres, where they are of different sizes and appear spaced along the axis at regular intervals (Fig. 29 b, c). The largest vesicular or fusiform dilatations are 5–8 μ in diameter and appear spaced at intervals of approximately 50–60 μ. Between them, a series of medium-size (3–4 μ) and small enlargements (1–2 μ) can be counted following closely upon each other, with spacings of 4–10 μ. Unilateral, irregular swellings and protrusions of the myelin sheath, which are clearly indications of damaged fibres, should not be mistaken for these symmetrical enlargements. No regularly occurring structures like sheath nuclei can be detected with phase contrast at the site of the enlargements. Although they are not preparation artefacts, it is possible that the fusiform enlargements bear a certain relation to surface tension forces which build up at periodic intervals in the naked myelin walls (without a neurilemma) of central nerve fibres below a certain diameter (129). Axon structures are not clearly discernible in the lumen of the fibres, but extremely fine fibrils can be seen coming out from their broken ends (Fig. 29 c). *A characteristic feature of these vesiculated fibres are the numerous connections with extremely fine fibres, which also show the same type of beaded or fusiform enlargements* (Fig. 29 d, e). The preservation of continuity between the finest fibres and the myelinated nerve fibres with fusiform swellings which they so closely resemble, could be ascertained in a large number of cases.

Examination of fresh frozen sections of the small myelinated nerve fibres with the electron microscope discloses that there are numerous heavily myelinated fibres with an external diameter of less than 1 micron (1–0.4 μ) (Fig. 28). Even taking into account the shrinkage due to desiccation and fixation, this would indicate that the size limits of 1 micron which have been set for small myelinated nerve fibres (58, 42, 91), are probably slightly lower. In transverse and longitudinal sections the fibres have a thick myelin sheath and the same type of axon filaments (100–200 Å thick) and vesicular axon structures described in the peripheral nerve fibres. The myelin sheath also exhibits the characteristic concentric laminated structure in suitable thin sections, with an average thickness of 80 Å for the single layers. Large pieces of lamellar membranes which are identical in all respects with the lamellar structures of peripheral nerve fibres, can be produced by dissociation of the sheath walls. Structures resembling the neurilemma membrane with associated collagen fibrils were not seen. Where the fibres break off in thick longitudinal sections, we frequently encounter dense bundles of axon filaments flowing out from the ends and partially enveloped by large thin membranes resembling myelin lamellae.

The numerous collateral branches and connections with other nerve fibres are studied best in fixed dissociation preparations or in thick (0.5–1 μ) frozen longitudinal sections.

Collaterals of different diameters (1–0.1 μ) arise from the main fibre without a perceptible break in the sheath. In the proximity of the sheath they are usually still heavily myelinated, but after a short distance they appear more translucent as the sheath becomes thinner. Dense fusiform enlargements are intercalated at variable distances along the course of the collateral fibres and generally mark the transition from the opaque central fibre to its two types of branches. These are:

(a) Fine fibres with a thin sheath and a dense, compact axon.
(b) Thin fibres, 1–0.1 μ in diameter, with an extremely tenuous sheath, characteristic vesicular enlargements and fine filamentous axon structures.

Most of these fibres correspond to the fine nerve fibres with fusiform enlargements already referred to in phase contrast preparations, and will now be described in detail.

2. *The submicroscopic nerve fibres of the cord with fusiform enlargements*

Since most of the nerve fibres belonging to this group are not clearly visible with the light microscope and their fine structure falls entirely within the range of submicroscopic dimensions, they have been designated as such to avoid confusion with other fibre types.

Osmium fixed dissociation preparations or longitudinal frozen sections of the lateral funiculus of the frog spinal cord abound in these characteristic fibres, which are thin enough to be nearly completely transparent to the electron beam. The fibres appear like flattened ribbons which reach lengths up to 50–80 μ and have a variable mean diameter ranging between 1 μ and 0.1 μ. All of them show fusiform or vesicular symmetrical enlargements spaced at regular intervals along the otherwise uniform fibre shaft (Figs. 30 to 43). These fibres can be identified as nerve fibres in view of their connection with the small myelinated fibres of the cord, their characteristic structure, and the localization among the nerve fibre bundles of the lateral funiculus.

Fundamentally, each fibre consists of a thin sheath formed by a single membrane of tubular configuration, which contains a few long thin filaments 100 Å in diameter and a variable number of other structures. Their water content must be high, or some of their components may have been lost in the process of preparation, since in the dried electron microscope preparations the thin sheath appears like a collapsed tube fitting loosely around the sparse internal filaments. The tubular character of the sheath can be clearly made out in shadowed preparations where certain more "solid" parts of the fibre have not been flattened out, and in transverse sections.

According to a closer study of their fine structure and an analysis of the distribution of fibre widths carried out on 500 fibres from the frog spinal cord, two general groups of fibres can be distinguished. One group comprises the fibres with a diameter of 0.5–1 μ (as measured in the dried, shadowed electron microscope preparations) and will be referred to here as fibres of medium size. The other group of "fine fibres" includes those with an average width of 0.1–0.5 μ. Transitions between the two types are of course numerous, and this classification was mainly adopted to facilitate description.

a) *The fibres of medium size with fusiform enlargements.*

The straight part or "shaft" of these fibres has a uniform width of 0.5–1 μ, with fusiform enlargements (1.5–0.8 μ wide and 3–1 μ long) spaced at regular intervals of 3–4 μ (Figs. 30–39). The fibres run extensive courses and specimens up to 80 μ long are not uncommon. *The tubular sheath is formed by a single membrane which is 60–80 Å thick, has a granular surface structure* (Fig. 37) *and is morphologically identical with the lamellar membranes of large peripheral fibres* (Fig. 8). Only that in this case we are dealing with a single tube instead of the multiple layers encountered in the thicker myelin sheaths.

The tubular sheath shows no visible line of junction where a conceivable fusion of the membrane ends could have occurred, and with the exception of minor transverse folds probably caused by drying, the sheath can be regarded as a seamless membranous tube. The granular structure of the membrane results from tightly packed rod-like granules which are 20–50 Å wide and 60–80 Å high, stain heavily with osmium and closely resemble the constituent granules of the myelin lamellae. At the edge of the sheaths, where the fine granules tend to separate, it can be seen that they are the sole constituents of the membrane, cemented together by some adhesive substance without the contribution of a basal membrane.

The fusiform enlargements contain several types of structures, which would mark these sites regardless of the concomitant sheath swellings. Opaque oval bodies, 1–0.5 μ long and similar to small cell nuclei are most common. These bodies seem to rest on top of the membrane and since the lighter zone of granular material surrounding them is apparently continuous with the sheath, a comparison with the Schwann cells could be assumed (Figs. 32–38). Many of them are semiopaque and reveal smaller aggregates of dense bodies in their interior, and what appears to be a wrinkled membrane. They occur single at regular intervals or collected in groups of 4–8 in large fusiform enlargements (Fig. 34). Dense fibrous structures, 400 Å thick, connecting rows of these nuclear bodies (Fig. 32), are also occasionally seen. Small vesicular bodies, 600–1800 Å in diameter, with indications of a folded membrane, are regularly found in the vicinity of the nuclear bodies (Figs. 33–36). It is difficult to determine, however, whether they are located on the surface or in the interior of the fibre. A more precise localization would help derive an analogy with the oval granula of the neurilemma membrane, or with the vesicular bodies in the axon of peripheral myelinated fibres.

The long, slender filaments run uninterrupted parallel courses throughout the entire lumen of the fibre and emerge free as dispersed bundles at the severed ends (Fig. 30). The filaments have an average diameter of 100 Å and appear invariably as solid fibrils with a regular beaded or nodose axial structure, as if they were formed by aggregation of minute granules aligned at 200 Å intervals. They are in all respects similar to the fine axon filaments of peripheral nerve fibres (Figs. 24, 26). There are fibres with only a few filaments (Figs. 33, 35), and others in which the compact filament bundles fill the entire axon and constitute the bulk of the fibre (Figs. 30, 31). The latter represent transitions to the typical unmyelinated fibres which will be described later.

In addition to the fine axon filaments one frequently encounters thicker (300–500 Å diameter) irregular varicose fibrils associated with the fibres (Figs. 34, 36, 38). It is difficult to determine if these varicose fibrils occur within the fibre together with the axon filaments, or are merely attached to the surface of the sheath. In shadowed preparations of the broken fibre end where definite indications of the axon filament's location in the interior of the fibre are obtained, these varicose bands fail to give clear-cut signs of their position within the fibre. They run single or in pairs, after emitting numerous contorted collateral branches along the entire length of the fibres (Figs. 36, 38), usually connecting the nuclear bodies. At some of the fusiform enlargements they also form wide-meshed nets or clusters. In view of their peculiar course and the association with the formations at the fusiform enlargements, these varicose bands might represent external sheath structures.

Finally, a limited number of fibres has been observed with a dense axonal strand (Fig. 39), 3000–4000 Å wide, running through the lumen of the fibre and joining the dense structures of the fusiform enlargements. The condensation of the axonal material in these fibres may indicate a transition to the collateral fibres of the small myelinated fibres, or simply be due to the action of the fixative.

b) *The fine submicroscopic fibres.*

The fibres belonging to this group have an average diameter of 0.1–0.5 μ, but a few of them barely measure 600 Å in certain parts and are over 50 μ long. Larger fusiform or vesicular enlargements (0.8–0.4 μ wide) occur at regular intervals of approximately 4–8 μ, alternating with smaller dilatations which are evenly spaced (1–1.5 μ) in successive groups of 3–4. The simplification of the structural organization achieved in these fibres has reached an extreme degree, since *they merely consist of a single layered tubular sheath containing only one filament* (Figs. 40–43), while most of the other structures are absent. The thin sheath formed by a single granular membrane displays the same fine structure and membrane thickness as the elementary sheath of the larger fibres. Here also, no sheath discontinuities or breaks can be detected in the straight portions or at the fusiform enlargements. The individual axon filament has an average diameter of 100 Å (Figs. 42, 43) but can become thicker (200 Å), and runs uninterrupted through most of the vesicular swellings, to attain about the same length as the fibre. It also shows the typical beaded axial fine structure of the axon filaments in larger myelinated nerve fibres. Opaque oval nuclear bodies (0.5 μ long) are present in the large fusiform enlargements, together with numerous vesicular bodies and granules of the type encountered in medium-size fibres. At the smaller vesicular enlargements simple flat distensions or contorted bundles of the axon filaments are seen. Connections of the fine fibres with those of medium size or with the collaterals of small myelinated nerve fibres are not common, probably because these delicate elements break off easily.

3. *The endings and terminal formations of the submicroscopic fibres and small myelinated fibres in the spinal cord*

Studies on the terminal formations of the fibres are limited to a number of preliminary observations on a few specimens in which structures resembling nerve endings were still in connection with the corresponding fibre.

The short collateral branches of the small myelinated fibres of the cord sometimes end abruptly in club-like formations 1 μ in diameter (Fig. 44). These oval endings show a coarse granular structure with a few dark bodies and seem to be bounded by a granular membrane. Unlike the fusiform enlargements of the collateral fibres which generally break off leaving a small stub attached to the enlargement, these club-like bodies appear to be closed, circumscript endings.

The medium size and fine submicroscopic fibres frequently seem to end in rounded off spherical or oval bodies also 1–1.5 μ in diameter (Figs. 45 a, b, c). The bodies are sharply bounded by a continuation of the sheath membrane which shows numerous folds but usually no openings or breaks. Several dense, round or oval bodies, with diameters ranging between 0.3 and 0.5 μ, can be seen in the interior of the globular formations, together with conglomerates of finer granules. The axon filament of the fibre leading to the spherical bodies usually ends among the granular masses. These terminal formations have been usually found free without contacting the surrounding structures, but they seem to be more numerous in preparations from regions adjacent to the gray matter of the spinal cord. Whether the described structures represent true nerve endings or mere vesicular enlargements of the fibres which have been severed off, can only be determined by further investigation. If they should correspond to the terminal boutons, the content of dense staining material which has been compared to the mitochondria (16, 105) might be related to the dark bodies found in the terminal formations described here.

4. *The pattern of organization of the submicroscopic nerve fibres*

From the preceding descriptions and the accompanying illustrations it can be concluded that the submicroscopic nerve fibres exhibit all the structural elements of ordinary myelinated fibres, arranged in an extremely compact and simple pattern which is determined by their limited size. Thus we find instead of multiple myelin layers a thin tubular sheath formed of a single membrane, but otherwise identical in fine structure and dimensions with the unit myelin lamellae of thicker fibres. The axon of these tiny fibres likewise contains thin long filaments which show the same fine structure as the axon filaments of larger fibres. Moreover, many structures analogous to the Schwann cells, the granular bodies associated with the neurilemma membrane and the axon constituents can be recognized among similar components of the submicroscopic fibres.

It is therefore permissible to regard these new fibres as submicroscopic prototypes of the larger myelinated nerve fibres, which confirm the main features of their structural organization as deduced from polarized light, X-ray diffraction and electron microscope studies.

An interpretation of the fusiform enlargements and other special structures revealed in the submicroscopic fibres must await further investigation, and would not affect the fundamental similarity existing between these fibres and the myelinated nerve fibres. Additional studies are also required to determine if these fibres represent specialized elements of the spinal cord, or structures common to the entire nervous system. The latter possibility seems more likely in view of their close similarity with the fine "varicose fibres" of light microscopy, which have been described as distinctive components everywhere in the nervous system.

3. *The unmyelinated nerve fibres of the spinal cord*

Ever since Remak's classical description (94), the unmyelinated nerve fibres have been recognized as special fibre types devoid of a clearly discernible myelin sheath with ordinary histological methods. Although polarized light studies suggest the presence of a tenuous myelin sheath even on the thinnest of these fibres (46, 61), there are other distinguishing features by which unmyelinated fibres can be identified. Remak's fibres are dense fibres of varying calibre, which seem to consist of very fine fibrils forming compact bundles, with adhering long slender nuclei and an enveloping thin neurilemma membrane (121). They are particularly abundant in the autonomic nervous system and in many cerebrospinal nerves (91). There are also unmyelinated fibres without a visible neurilemma called naked axons, occurring in large numbers in the gray matter of the brain and spinal cord (91). The unmyelinated fibres studied here were isolated mainly from the gray and white matter of the frog and rat spinal cords, since these fibres are often thin enough for electron microscopy and easy to recognize. Dissociation preparations and sections of sympathetic trunks, which were also investigated, contain many contaminating connective tissue structures in addition to the typical Remak fibres, and are therefore less suitable for examination.

In fresh longitudinal sections through the lateral funiculus of the rat spinal cord where the fine unmyelinated fibres are especially numerous, these straight, dense fibres can be easily picked out with the phase contrast microscope. The larger ones (Fig. 29 f) are 2–4 μ wide and several hundred microns long, exhibit fine longitudinal striations and seem to have no sheath. Instead, irregular long dense particles and occasional larger bodies resembling nuclei adhere to their surface. The finer fibres (0.2–1 μ) have a typical smooth, straight, wire-like appearance (Fig. 29 g), while the finest ones are close to the limit of visibility and difficult to differentiate from the fibrils with fusiform enlargements.

Examination of isolated osmium fixed unmyelinated fibres with the electron microscope discloses straight, cylindrical cords, 0.5–2 μ in diameter, which are nearly completely opaque except in certain regions, where flattening or loosening of the compact bundles has occurred. In these areas (Figs. 46–49) it can be clearly seen that *the fibres consist of uniform filaments, approximately 100 Å in diameter and of indefinite length, which are tightly packed together.* The single filaments have a typical nodose appearance throughout their entire length suggesting an axial periodicity of approximately 200–300 Å, and run perfectly independent, straight courses without lateral branching or fusion despite the close packing. They seem to be held together by some diffuse binding substance which would account for the extremely compact appearance of the fibres. *All of the fibres examined show a thin sheath consisting of a single granular membrane approximately 60–100 Å thick,* which envelops the dense bundle of filaments. The membrane is not readily apparent in some of the fibres which seem to be nothing more than "naked" fascicules of filaments entirely devoid of a sheath (Fig. 48). Closer study, however, will reveal remnants of the tenuous sheath in the form of granular membrane patches at the edges of the fibre (Fig. 47). The membranes exhibit the same type of granular fine structure (consisting of tightly packed rod-like granules 60–90 Å long and 20 Å wide) and tubular configuration which are common to all kinds of nerve fibres. Long, opaque bodies resembling nuclei (Fig. 46) appear attached to the membrane of the larger fibres at regular intervals, and are especially prominent in the typical Remak fibres of sympathetic nerves. The smallest fibres, 0.2–0.4 μ in diameter, represent true transition forms (Figs. 30, 31) with their numerous axon filaments and characteristic fusiform enlargements.

The thin filaments are the sole components of the axon in the majority of these fibres, since only small amounts of a finely dispersed granular material can be found among the solid filament bundles fitting tightly together without intervening free spaces. No transverse periodic structures, which might possibly result from lateral register of the axial pseudoperiodicity of the single filaments, could be detected in the smaller fibres.

According to these findings, the so-called unmyelinated nerve fibres show the same submicroscopic structural pattern of myelinated fibres, since they too consist of fine axon filaments surrounded by a membranous sheath. The presence of an extremely thin sheath only 60–100 Å thick contributes little, however, to the structure of a fibre consisting almost exclusively of compact fascicules of axon filaments. Thus, while the term "unmyelinated" may be essentially inaccurate, it still denotes *a comparative* *paucity of myelin sheath and a preponderance of axon material which are characteristic features of these fibres.* The classical terminology was therefore retained here to avoid confusion, although the general designation of "Remak fibres" or "C fibres" (42) for all unmyelinated nerve fibre types might eventually prove to be more convenient.

DISCUSSION

1. *Analysis of the preparation techniques*

Since most of the observations recorded here were made on thin (1 μ) and ultrathin (0.1–0.3 μ) frozen serial sections of fresh nerve, several of the critical factors involved in the freezing-sectioning technique will be discussed first. The structural changes and artefacts resulting from the use of fixatives, the mounting and drying of the specimens, and the effects of electron bombardment will also be briefly evaluated.

a) *Freezing-sectioning of fresh nerve.* In the method described, satisfactory preservation of nerve fine structure is due primarily to the favourable conditions for rapid freezing and sectioning offered by small pieces of fresh nerve not exceeding a few millimeters in volume. Since the thin sections are collected on liquid surfaces, or in a still-frozen state on dry polished surfaces attached to the knife edge, followed by direct transfer to the supporting collodion films, specimen distortion is reduced to a minimum. The temperatures of —40° C which can be reached by cooling with carbon dioxide, and of —100° C when working with liquid air stages, are adequate for producing vitrification of protoplasm (64—70). Even better conditions are operative, if the fresh nerve is dehydrated by immersion in sucrose or glycerine solutions of suitable concentration prior to freezing.

However, vitrification of nerve protoplasm is not in itself the decisive factor, since a number of observations performed on deep-frozen nerve sections indicate that limited interstitial ice formation may not necessarily be detrimental. In still-frozen sections of fresh nerve collected on undercooled glycerine and examined with the phase contrast microscope before thawing had occurred, longitudinal columns of ice were found lodged in the interstices between the fibre bundles, while the fibres were free of ice and probably in an undercooled state. As has been suggested earlier (39), in frozen tissues we probably have a combined undercooling of small masses of "bound water" (74) in the macromolecular protein complexes of protoplasm, in addition to more extensive ice formations of the "interstitial water." In a general way, one can conceive of this interstitial water which permeates the entire tissue as a *potential natural "embedding medium"* which can be made to solidify by freezing. The three-dimensional submicroscopic framework of congealed interstitial water which is then formed along natural crevices and cleavage boundaries imparts the rigidity necessary for sectioning. Once sectioning is accomplished, one gets rid of this ideal embedding and hardening medium simply by melting the ice at the most convenient moment. The interstitial ice formation along submicroscopic boundary planes may also prove advantageous for ultrathin sectioning, by inducing separation along these potential planes before the "splitting" action of the knife edge brings about actual cleavage.

If the separation of the thin tissue slice from the frozen block is followed by immediate thawing and spreading on the comparatively warm liquid surfaces of the collecting reservoir, the dangerous formation of ice during devitrification can be entirely avoided. In certain cases where thawing might prove detrimental at this stage, the sections can be kept in a still-frozen state by collecting on undercooled liquids (concentrated glycerine, glycol, sucrose, etc.), and then subjected to the described freezing-drying procedures.

The freezing-sectioning method has been tested on a variety of fresh and fixed tissues and microorganisms (normal and bacteriophage infected bacteria, spermatozoa etc.) proving conclusively that *satisfactory ultrathin serial sectioning of frozen tissues can be carried out routinely* (39). An important limitation of the freezing-sectioning technique is determined by the fact that structural relations involving larger areas of tissue are not adequately preserved in unembedded frozen sections. In view of the required small tissue pieces and the consequent limited areas available for examination, *frozen sections will always have to be supplemented by corresponding sections of embedded material.*

The described freezing-sectioning techniques are obviously still in a preliminary stage of development, and considerable improvement in preservation of fine structure can be expected from the systematic use of lower temperatures, protective dehydrating treatment of the fresh tissues, and superior freezing-drying and specimen mounting techniques.

b) *Effects of fixation, drying and of the complementary preparation procedures.* The use of thin fresh frozen sections which were air-dried or subjected to the modified freezing-drying procedures has made it possible to examine unfixed sections directly with the electron microscope. Although these fresh frozen-dried sections still show numerous drawbacks like contamination with precipitated salts and low stability under the electron beam, they have been of great value in establishing the *fundamental similarity between structures revealed in fixed material and those seen in fresh, untreated nerve.* Osmium tetroxide has proved to be the best fixative, provided it is applied in buffered solutions and for suitable periods of time. Application of osmium in vapour form directly on the thin frozen sections gives superior preservation of the axon structures, while proper osmium fixation of whole nerve yields better preparations of the myelin sheath. Reaction products of osmium tetroxide which are deposited in an oriented way in the sheath (101—103) must be kept constantly in mind, since they may give rise to erroneous interpretations of structure. In the case of the crystalline osmium structures closely resembling myelin lamellae, recognition was possible by the recorded crystal interference phenomena and electron diffraction patterns, but identification of amorphous osmium deposits is more difficult.

Parallel phase contrast and electron microscope studies on the effects of enzymatic digestion and extraction of fresh nerve sections must be carried out on a large number of specimens. Since the fresh frozen sections are only supported by a thin collodion membrane, these treatments result in extensive release of different structures which contaminate each other and obscure original relationships. Enzymatic digestion and extraction of whole nerve often give better results in this respect.

Distortion of the structures, introduced by drying, can be minimized by subjecting the fresh or fixed sections to the described freezing-drying procedure. In these frozen-dried sections shrinkage is reduced and many details of their three-dimensional structure are still preserved. The dissociation techniques are of considerable value in yielding whole, well preserved specimens of objects like the submicroscopic nerve fibres.

Adequate localization and interpretation of the structures demand, however, parallel controls with alternating serial sections of the same tissue block. The effects of electron bombardment are particularly evident in unfixed sections, but generally negligible in well fixed preparations. By using low electron optical magnifications and a small condenser aperture of 200 μ, both the beam intensity and the size of the irradiated specimen area can be suitably reduced.

2. *Recent electron microscope studies of nerve fibre structure*

During the past two years a number of important contributions have been made to the study of nerve fibre structure with the electron microscope. In 1949–1950 Schmitt (106, 107) examined the fibrous constituents of formalin-fixed and unfixed axoplasm of the giant fibres of the squid and of the marine annelid *Myxicola*, showing that the fixed axons contain nodose filaments of indefinite length and having widths ranging between 75 and 200 Å. In unfixed axon material, filaments 100–150 Å wide, with relatively smooth edges, were found. The dense-edged fibrils described previously as "neurotubules" by De Robertis and Schmitt (30, 31) were considered by Schmitt to be derivates of the connective tissue sheath which may appear as contaminants of isolated axon preparations. Schmitt and Geren (114) examined thin sections of osmium and formalin fixed squid, frog, rat nerves and human sympathetic nerves with the electron microscope, and found the same type of axon filaments having diameters ranging from 100 to 200 Å and indefinite length. These filaments exhibit axial discontinuities spaced fairly regularly at intervals of 200–350 Å which gives them a nodose appearance. Similar filaments were described subsequently in thin sections through the axon of osmium fixed nerve by Baud (8), Duncan and Antes (33), and Bretschneider (24).

Membranous structures composed of several layers of thin membranes showing a uniform thickness were described in fragmentation preparations of osmium fixed guinea pig nerves by Sjöstrand (118). Hartmann (48) examined thin sections of osmium fixed spinal cord of the rat embedded in methacrylate, and describes concentrically arranged placodes in the myelin sheath of the nerve fibres. These placodes of varying electron density, 60–120 Å in thickness, 540–1150 Å wide and 900-1700 Å long, as shown in his micrographs, closely resemble the oriented osmium deposits regularly found in the myelin sheath. They can be clearly distinguished from the concentric laminated structure of the sheath, although their oriented deposition bears a certain relation to the laminated fine structure of the myelin sheath. According to Schmidt (102, 103) the black reaction product of the osmic acid is deposited in an oriented way in the myelin sheath, abolishing the birefringence of lipoids but introducing a weak positive uniaxial birefringence with radially oriented axis, which partially compensates for the loss of lipoid birefringence.

By means of a special ultrathin sectioning method, Sjöstrand (119) has been able to demonstrate the concentric laminated structure of the sheath very clearly in osmium fixed guinea pig sciatic nerves. Sjöstrand describes periodic striations with a period of 50–80 Å, presumably corresponding to a concentric arrangement of the membranes in the sheath, and thus confirming the dimensions of the concentric laminated structure described previously by the present author (37). Rozsa, Morgan, Szent-Györgyi and Wyckoff (99) have examined thin sections of formalin fixed and methacrylate embedded sciatic nerves of the rabbit, describing an undulating fibrillar axon structure running parallel to the fibre axis and composed of filaments about 200 Å in diameter. According to these authors the myelin sheath is covered by a neurilemma about 500 Å thick, and the nerve fibres are bound together by connective tissue filaments which have the characteristic cross striations of collagen fibrils. The myelin sheath appears severely damaged by the extraction of lipids in their preparations and is described as "an anastomosing lamellar net or cage." Conical partitions of the sheath, corresponding to the clefts of Schmidt-Lantermann, are discernible in the fibres.

From this review it can be seen that the main structural features of myelinated fibres described previously (36, 37) have been largely confirmed by various electron microscope investigations performed on different nerve types.

3. *The structure of the myelin sheath*

In addition to more detailed observations on the lamellar structures described previously in osmium fixed whole nerve (37), further information has been obtained by the study of fresh nerve sections and of the simplified relations existing in the submicoscopic nerve fibres.

The myelin sheaths of thin fresh nerve sections which have been frozen-dried or fixed with osmium vapours separate into multiple membranous layers having the same thickness and granular fine structure as the fixed myelin lamellae. The stratified layers isolated from thin sections of osmium fixed whole nerve show the best degree of preservation, appearing as flexible membranes, approximately 80 Å thick, with a characteristic granular structure and piled up on top of each other. The number of concentrically arranged unit lamellae or membranes varies according to the thickness of the myelin sheath, and ranges from over a hundred layers in the sheaths of larger myelinated nerve fibres to the extreme of one single layer in the submicroscopic nerve fibres and in the unmyelinated fibres. The single membrane of these tiny fibres is 60–90 Å thick, forming a tubular, seamless sheath which is continuous with the nuclear structures resembling Schwann nuclei in the fusiform enlargements. It has a fine granular structure and dissociates at the edges into rod-like granules 20 Å wide and 60–90 Å long. The same type of granular disintegration is observed in the membranes of the myelin sheath, particularly marked after trypsin digestion, where the unit lamellae separate into granules of similar dimensions without the apparent contribution of a basal supporting membrane. Alcohol extraction of whole nerve produces compact residual masses in the sheath which upon sectioning yield numerous smooth lamellae only 30–40 Å thick. On the other hand, alcohol extraction of the thin fresh nerve sections results only in massive release of numerous small foamy droplets, leaving a cribriform lamellar network behind which is reminescent of the "neurokeratin" network. This gross disorganization of the sheath cannot be evaluated as representing its true structure after extraction of the lipids. Therefore, while indirect evidence indicates that the myelin membranes are composed of compactly bound, radially oriented, rod-like lipid particles arranged into concentric layers in the sheath by a protein framework of some kind, further studies are required to establish the details of this structure. In this respect the digestion of fresh nerve sections with specific enzymes appears promising, in order to break up the lipid components in the membranes without drastic alteration of the protein structures.

The characteristic folding and warping of the myelin lamellae produced by ultracentrifugation of the nerve fibres can be interpreted as indicating that the membranes are preformed elements of the sheath, and not some kind of reproducible "equivalent" structures resulting from the action of certain preparation techniques. Whether this extremely uniform stratification of multiple membranes is present in the living nerve fibre, or possibly fluctuates according to its functional state (distances between layers becoming larger due to swelling, for example), still remains to be investigated. Eventually, the correlation of permeability changes during nerve impulse conduction with possible ultrarapid structural alterations in these membranes may become a central problem (40).

Improved preparation techniques have demonstrated clearly the laminated fine structure of the sheath in longitudinal and transverse sections of different nerve types. As described previously, it consists of a system of parallel, uniform dark lines separated by lighter spaces, with a regular period ranging from an average of 60 Å in frog nerves to 80 Å in nerves of the rat, cat and rabbit. In suitable preparations, direct transitions can be observed between the laminated structure and its separation into thin lamellar ribbons, indicating that it corresponds to the concentric arrangement of the membranes in the sheath.

From the accumulated evidence available, it can therefore be concluded that *the myelin sheath is predominantly built up of a varying number of concentrically arranged uniform layers or membranes approximately 80 Å in thickness.* This is in general agreement with the results of polarized light (46, 102, 104, 108, 111) and X-ray diffraction studies (112), which indicate that the myelin sheath consists of concentrically arranged lipid-protein layers of these dimensions. Recently, J. B. Finean (personal communication) has recorded spacings of 80 Å and 160 Å in the X-ray diffraction patterns of frog nerves which have been frozen and then thawed before drying. He comes to the general conclusion that fresh nerve myelin consists of a lipid-lipoprotein system forming concentric layers around the axon. The "labile" lipid of this system separates easily from the main structure forming separate phases with poor orientation, while the "residual" lipoprotein retains the highly ordered laminated structure present in the fresh sheath, with slight variations according to the treatment. These findings suggest that we may expect to achieve still better preservation of the original myelin sheath fine structure by improving the preparation techniques in order to minimize the induced changes. Further essential information on the ultrastructure of the myelin sheath in the natural wet state could perhaps be obtained by performing specially devised polarized light and X-ray diffraction studies directly on the fresh nerve sections of suitable thickness, instead of on whole nerve fibres. The possible existence of reinforcing structures at the incisures or around the internal wall of the sheath has not been demonstrated yet, and the reality of the described crevices (37, 48) in the myelin sheath is likewise still uncertain.

The oblique conical partitions or clefts occurring symmetrically in the sheath are very distinct in thin frozen sections and correspond to the clefts of Schmidt-Lantermann which are also seen in the living fibre (76). Demonstration of these oblique clefts in fresh frozen sections and in embedded nerve (99) with the electron microscope supplies further evidence that they may be natural gaps present in the myelin sheath. In frozen sections they are generally empty, but some of them contain a granular or fibrous material which may be related to the complicated formations described in the incisures by silver impregnation techniques (87, 88).

The structure of the neurilemma membrane and of the associated Schwann cell nuclei has already been described and is best represented in the smallest nerve fibres. From a cytochemical point of view the Schwann cells are among the most interesting components of the nerve fibre, and their comparatively easy preparation in an unfixed state by means of the replica-adhesion and freezing-sectioning techniques should facilitate studies of this kind. If it can be shown that the slender varicose bands found in the submicroscopic nerve fibres of medium size (Figs. 36, 38) are attached to the surface of the sheath, they might represent protoplasmic expansions of the Schwann cells. The oval bodies (Figs. 19, 20) which are found in the vicinity of the Schwann cells probably correspond to the Reich granula (93); their origin and functions are unknown.

As has been pointed out before (38), *a clear distinction can be made between the real membranous layers of the myelin sheath and the crystalline lamellar structures.* By application of the electron diffraction techniques and observation of the characteristic crystal interference phenomena, these crystalline lamellae can be picked out among the associated myelin lamellae. The fact that they so closely resemble each other, together with the demonstration of similar crystalline lamellae in osmium fixed lipid extracts of whole nerve, furnishes indirect evidence that the crystalline structures may be osmium-lipid compounds formed during the process of fixation. Until reliable identification of these crystalline lamellae is possible by correct interpretation of the recorded cross-grating and Kikuchi line electron diffraction patterns (Figs. 11, 13), they can only be regarded as osmium fixation products in the preparations. Their presence, however, calls attention to the possibility of performing electron diffraction studies on structures like the myelin lamellae which are of suitable thickness and configuration for these investigations. Natural biological structures could be enhanced and stabilized by selective deposition of heavy atoms acting as "electron stains" (47), and the electron diffraction patterns recorded under these conditions would have the advantage of allowing direct correlation with the corresponding electron microscope image of the object. With the described arrangement, the RCA EMU electron microscope can also be used as a high resolution electron diffraction camera (85) to record spacings up to 10 Å, which would thus overlap with the limit of resolving power attainable with the electron microscope.

4. *The structure of the axon*

The use of fresh frozen sections has given better preservation of the axon structures and made it possible to follow their course over longer areas in thick sections. *In the different types of peripheral and central nerve fibre sections examined after freezing-drying or fixation with various reagents, the axon consists of uniform nodose filaments, 100–200 Å in diameter, having indefinite lengths and a predominant longitudinal orientation.* Their number varies from the thousands present in axons of larger myelinated fibres to the single filaments found in fine submicroscopic fibres with fusiform enlargements. Unmyelinated nerve fibres which consist almost entirely of compact filament fascicules represent special fibre types. The axial discontinuities spaced regularly at intervals of 200–300 Å are responsible for the nodose appearance which is common to the filaments of all fibres, although it is not apparent in unfixed filaments. The axon filaments always appear solid, and double-edged, tubular structures of the type described by De Robertis and Sotelo (32) were not seen, except in certain formalin fixed or embedded sections, where contamination with lipids was unmistakeable, since addition of alcohol or acetone abolished the tubular appearance.

All of these observations were made on dried material and the question arises whether these filaments are really present in the living fibre or were produced by the effects of freezing, sectioning and drying. Polarization optical studies indicate the presence of elongate particles with positive form birefringence in the axon of squid giant fibres (13, 14). Schmitt (106) has pointed out that these properties may be due to thin axon filaments of this type, since electron microscopy has revealed thin filaments in objects like muscle, flagella and sperm tails with manifest positive form birefringence.

Fibrous structures are not visible in the axons of thin longitudinal sections of fresh nerve examined with the phase contrast microscope, but the formation of structures resembling neurofibrils can be followed during fixation of the sections. Under the electron microscope, these neurofibrils correspond to long bundles formed by lateral aggregation of the thin filaments. From these and other observations (106) it seems probable that the neurofibrils represent bundles of axon filaments, and neurofibrils have been seen in living nerve cells (127, 120, 63). Moreover, earlier ultracentrifugation experiments on spinal ganglion cells (9, 59, 75) show that the neurofibrils may be displaced and concentrated. The described ultracentrifugation of whole nerve, followed by electron microscope examination, also indicates that the axon filaments can be displaced and sedimented without loss of their characteristic filamentous structure. If the filaments were precipitated from the fibrous axon protein, the production of artificial concentration areas in the fresh axon by ultracentrifugation would probably lead to corresponding modifications in the fine structure of the precipitate. Instead, one merely finds here the dislocation, contortion and twining of the bundles formed by individual filaments which would result if *preformed filamentous structures in the axon* were subjected to the effects of ultracentrifugation.

In the case of the submicroscopic nerve fibres, where a single filament of 100 Å is enclosed in a membrane barely 90 Å thick, it is difficult to explain the clear-cut, reproducible electron microscope pictures as coagulation effects, since such an intimate association of structures could hardly be expected to precipitate consistently in the same regular fashion. The suggestion of Péterfi (84) that the fresh axon is a rodlet sol capable of forming elongated particles under slight chemical provocation is still tenable, and the possible effects of ionic strength, pH and enzymes (106) on this process are important. But one could go even further and conceive of the axon filaments as stable fibrillar elements capable of active contraction under certain conditions, like the filaments of the cilia and sperm tails which they resemble in some respects. Our knowledge of the axon filaments is still fragmentary, and more evidence is required to establish that they really exist in the living nerve fibres.

A greater concentration of filaments around the internal sheath wall was regularly observed in fresh nerve sections, but a well defined fibrous membrane of the type described earlier as the axolemma (37) in osmium fixed fibres could not be detected. Indications of an axolemma membrane were also absent in the smallest nerve fibres. It is therefore probable that the axolemma membrane, described as a dense net of interlaced filaments coating the internal sheath wall, represents the artificial accentuation of a naturally occurring greater accumulation of the axon filaments around the internal wall.

The clear vesicular bodies (Figs. 23, 25) found regularly among the filaments of the axon in fresh nerve sections fixed with osmium may possibly represent axon components, provided contamination with sheath structures can be definitely ruled out.

5. *The general pattern of nerve fibre organization*

The fundamental similarity of the elementary structures revealed in the different nerve types makes it possible to recognize a pattern of organization common to myelinated fibres of all sizes and to the unmyelinated nerve fibres. Thus, the single membrane forming the sheath of the submicroscopic fibres can be regarded as the prototype of thicker myelin sheaths composed of multiple membranes. In this case, the arrangement of thin walled tubes fitted tightly into each other which was proposed tentatively in a schematic diagram of the myelinated fibres (37), would be derived from the simple tubular membranes of the submicroscopic fibres. The longitudinal disposition of the filaments within the axon is likewise apparent in all types of nerve fibres. Equivalents of the Schwann cells, neurilemma membranes and associated structures are also represented in each fibre type throughout the whole spectrum of nerve fibre size. As a result of the submicroscopic dimensions of these basic units, an extraordinary degree of compactness and miniaturization is achieved in the organization of the spinal cord fibre tracts, for example. Here the substructure begins with the simplest combination of two elementary components only 100 Å thick, and increasingly complex formations are built up by systematic repetition of the same pattern, like in the case of gradual transitions from submicroscopic nerve fibres to large myelinated fibres.

There are of course special formations in the smallest fibres which have no direct counterpart among larger fibres. Nevertheless, the fusiform enlargements of the small fibres suggest a certain analogy with the nodes of myelinated fibres in view of their constant occurrence and fairly regular spacings. From another point of view the existence of these submicroscopic fibres poses an interesting problem to the neurophysiologist, because recording the action potentials from an individual fibre 0.1 μ in diameter with suitable micro-electrodes would actually amount to detecting the activity of a single membrane-filament system, which presumably are basic elements of all nerve fibres. The myelin lamellae are similar to the unit discs of the retinal rod outer segments of guinea pigs described by Sjöstrand (117), and to the discs (60) or bladders (1) of the chloroplasts. They may also resemble the lipoid membranes of many cells (100), and thus represent a general type of submicroscopic structure.

Further progress in the study of nerve fibre structure with the electron microscope will depend primarily on improved preparation techniques, and on the systematic application of other methods of ultrastructural analysis.

SUMMARY

I. Thin serial frozen sections of fresh peripheral and central myelinated nerve fibres from the frog, rat, cat and rabbit, in addition to spinal cord segments of frog and rat, were examined with the electron microscope after freezing-drying or fixation with osmium. Supplementary investigations included parallel observations by phase contrast microscopy and examination of thin sections of embedded nerve, replica-adhesion and dissociation preparations.

1. The following structures are described in the internode portion of the peripheral myelinated nerves:

a) The myelin sheath of fresh nerve separates into layered membranes which closely resemble the myelin lamellae of fixed whole nerve. Lamellar membranes of this type with an average thickness of 80 Å and a granular fine structure have been found in all nerve types. By means of ultracentrifugation of fresh whole nerve these laminated membranes can be warped in a reproducible fashion, indicating that they are probably preformed structures in the sheath. Dissociation of the fresh, unfixed membranes into rod shaped granules 60–80 Å long is produced by trypsin digestion. The concentric laminated structure of the sheath, consisting of individual layers approximately 80 Å thick, could be confirmed in all nerve types examined.

b) Oblique clefts corresponding to the Schmidt-Lantermann incisures were observed in the myelin sheath, and further structural details of the neurilemma membrane, its associated fibre bundles and the Schwann cells noted. Numerous opaque, oval bodies (0.5–1 μ) with a granular internal structure were found close to the Schwann cell.

c) An arrangement is described for obtaining successive electron diffraction patterns and electron micrographs from restricted areas of the same object in the specimen holder of an RCA EMU electron microscope, without breaking the vacuum. With this arrangement, characteristic cross-grating and Kikuchi line patterns have been recorded from crystalline structures resembling myelin lamellae in osmium fixed preparations. The evidence available suggests that these crystalline lamellae are osmium products formed in the nerve during the process of fixation.

d) The axon of fresh nerve fibre sections which have been frozen-dried or fixed, contains filaments of indefinite length, with a diameter of 100–200 Å and regularly spaced axial discontinuities. These filaments show a predominantly longitudinal arrangement within the fibre and can aggregate to form compact bundles resembling neurofibrils. Dense oval corpuscles and clear vesicular bodies are also seen in the axon.

2. In thin sections and dissociation preparations from the lateral funiculus of the frog and rat spinal cords, new types of submicroscopic fibres were found which have diameters ranging between 0.1 and 1 μ, and can be identified as nerve fibres by their connection with larger myelinated fibres and their fine structure. These fibres have regularly spaced fusiform enlargements occupied by dense bodies resembling Schwann nuclei, and consist of a thin sheath formed by a single tubular membrane, which contains long filaments 100 Å in diameter and a variable number of other structures. The finest fibres consist of a single membrane and only one central filament. The sheath membrane of these fibres is 60–80 Å thick and exhibits the same granular fine structure as the layered membranes of myelinated nerve fibres.

3. Unmyelinated nerve fibres from the spinal cord and the sympathetic chains of frog and rat are constituted by compact bundles of nodose filaments, having a diameter of 100–200 Å and indefinite length, and are invested with a single granular membrane approximately 60 100 Å thick. Dense slender bodies resembling nuclei are attached to these membranes.

II. The findings presented here indicate that the structural elements of all types of nerve fibres are fundamentally similar, and point to a common pattern of organization in myelinated fibres of all sizes and in the so-called unmyelinated nerve fibres.

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TEXTS TO THE FIGURES

Fig. 3. Modified freezing microtome for ultrathin sectioning with electric motor drive. The freezing stage is clamped in the specimen holder of a Spencer rotary microtome, and is connected with the carbon dioxide container through a suspended pressure hose. The binocular microscope, special knife holder with attached collecting trough and the freezing cup are also shown.

Fig. 4. Glass knife in the special holder described by Latta and Hartmann, with collecting trough clamped on by means of a spring, used for cutting ultrathin frozen sections.

Fig. 5 a. Close-up view of the frozen, fixed but unembedded nerve block attached to the plexiglass disc with filter paper. Notice band of serial thin sections floating on the liquid of the collecting reservoir. 1:4.

Fig. 5 b. Enlarged view of fresh frozen sections (1 μ) cut from a nerve piece. Notice the two sections (arrow) floating on the Ringer solution in the trough, between knife edge and an electron microscope screen with central slit. 1:8.

Fig. 6. Phase contrast micrograph showing part of a whole transverse section (1 μ) through fresh nerve picked up on Ringer (see Fig. 5 b). Notice preservation of section integrity and of the fibre structure. Magnification: 200 ×.

Fig. 7 a. Phase contrast micrograph of 1 μ thin transverse sections of frog sciatic fibres mounted on collodion-covered electron microscope grid in drop of Ringer solution. Notice homogeneous myelin sheath and clear axon. 600 ×.

Fig. 7 b. Electron micrograph of a nerve fibre from preparation shown in Fig. 7 a with dense net of axon filaments, several opaque axon bodies and a few strands of collagen fibres at the periphery of the myelin sheath. Osmium vapour fixation. Unshadowed. Magnification: 15 000 ×.

Fig. 8. Pile of 8 myelin lamellae dissociated from the myelin sheath of cat motor roots. Dow Latex calibration particles (2 600 Å diameter) have been added to determine the thickness of the unit lamellae. Notice characteristic granular surface structure of the lamellae. Osmium fixation. Pt/Pd shadowed. Magnification: 25 000 ×.

Fig. 9. Pile of "osmium lamellae" from a dissociation preparation of osmium fixed rat sciatic nerve. Notice the wavy dark lines corresponding to extinction contours, and the single, smooth lamellae with angular edges. Pt/Pd shadowed. 20 000 ×.

Fig. 10. Pile of "osmium lamellae" from a dissociation preparation of osmium fixed frog sciatic nerve. Irregular dark areas corresponding to extinction contours are seen. Collodion supporting film. Unshadowed. Magnification: 20 000 ×.

Fig. 11. Cross-grating electron diffraction pattern recorded from the lamellae pile of Fig. 10, using the specimen in the object position of an RCA EMU electron microscope (50 kV) fitted with a condenser aperture of 200 μ and an intermediate lens.

Fig. 12. Thick pile of "osmium lamellae" from a dissociation preparation of osmium fixed frog sciatic nerve. Approximately 40 transverse striations can be seen, corresponding to the edges of the lamellae. Unshadowed. Magnification: 24 000 ×.

Fig. 13. Kikuchi line pattern recorded from the lamellae pile of Fig. 12, using the specimen in the object position of an RCA EMU Electron microscope (50 kV) fitted with a condenser aperture of 200 μ and an intermediate lens.

Fig. 14 a. Fibre from an ultracentrifuged whole sciatic nerve with detached myelin sheath collected at the bottom in a folded serpentine structure. Subsequently osmium fixed. Phase contrast. Magnification: 800 ×.

Fig. 14 b. Fibre from an ultracentrifuged whole sciatic nerve with detached and folded myelin sheath. Phase contrast. 400 ×.

Fig. 15. Electron micrograph of a thin longitudinal frozen section through osmium fixed ultracentrifuged rat sciatic fibres of the type shown in Fig. 14 a, b. Notice characteristic corrugation and creasing of the thin myelin lamellae. Unshadowed. 12 000 ×.

Fig. 16. Ultrathin (0.1 μ) transverse frozen section through the myelin sheath of osmium fixed frog sciatic nerve fibre, showing the concentric laminated structure. Over 40 parallel lines with an average spacing of 70 Å can be counted here. Unshadowed. Magnification: 140 000 ×.

Fig. 17. Bundles of thin smooth fibrils from a longitudinal frozen section of osmium fixed rat sciatic. The smooth fibrils, 100–200 Å in diameter, probably correspond to the elastic elements of the outer sheath layers. Pt/Pd shadowed. Magnification: 24 000 ×.

Fig. 18. Bundle of fibrils with characteristic collagen periodicity from the outer layers of rat sciatic nerve fibres. Longitudinal frozen section of fresh nerve subjected directly to the modified freezing-drying procedure and reinforced with aluminum film. Pt/Pd shadowed. 20 000 ×.

Fig. 19. Longitudinal frozen section through the neurilemma membrane close to the Schwann cell. Osmium fixed rat sciatic. Dark oval bodies are seen adhering to the membrane. Pt/Pd shadowed. Magnification: 18 000 ×.

Fig. 20. Replica-adhesion preparation of air-dried isolated frog sciatic nerve fibre, showing the surface structure of numerous oval bodies in the vicinity of a Schwann cell. Pt/Pd shadowed. 12 000 ×.

Fig. 21 a. Phase contrast micrograph of fresh longitudinal section (1 μ) through frog sciatic nerve fibres. Schmidt-Lantermann clefts can be clearly seen in the largest fibre. Magnification: 600 ×.

Fig. 21 b. Phase contrast micrograph of fresh longitudinal section (2 μ) through a rat sciatic nerve fibre. 2 pairs of Schmidt-Lantermann clefts following closely upon each other can be seen. 800 ×.

Fig. 22. Electron micrograph of a Schmidt-Lantermann cleft from a fresh longitudinal frog sciatic nerve section. Notice oblique, empty gap in the sheath, nearly extending into the axon. Osmium vapour fixation. Pt/Pd shadowed. Magnification: 8 000 ×.

Fig. 23 a. Longitudinal frozen section (1 μ) of fresh frog sciatic nerve fibre mounted on the collodion supporting film of an electron microscope screen, in Ringer solution. No structures are visible in the clear axon. Phase contrast. Magnification: 600 ×.

Fig. 23 b. Electron micrograph of a longitudinal section through nerve fibre of the same preparation shown in Fig. 23 a. Notice the long strands of predominantly longitudinally oriented filaments in the axon, also the rows of clear vesicular bodies. Bundles of collagen fibrils are attached to the outer layers of the sheath. Osmium fixation. Pt/Pd shadowed. Magnification: 16 000 ×.

Fig. 24 a. Longitudinal frozen section (1 μ) of fresh frog sciatic nerve fibre mounted in Ringer sol. on the supporting film of an electron microscope screen with central slit. Faint indications of a fibrous structure in the axon are beginning to appear under the action of osmium vapours. Phase contrast. Magnification: 800 ×.

Fig. 24 b. Electron micrograph of a longitudinal section through a nerve fibre of a similar preparation as in Fig. 24 a. In these thick sections the course of the axon filaments can be followed over long stretches. Notice vesicular bodies with indications of wrinkled, collapsed membranes. Osmium fixation. Pt/Pd shadowed. Magnification: 19 000 ×.

Fig. 25. Segment of an axon from a longitudinal fresh frozen section (1 μ) of frog sciatic fibre fixed with osmium. Semiopaque oval body with membrane streamers is seen together with clear vesicular bodies among the axon filaments. Pt/Pd shadowed. Magnification: 19 000 ×.

Fig. 26. Axon segment from a longitudinal fresh frozen section (0.5 μ) of frog sciatic fibre fixed with osmium. Notice parallel course of longitudinally oriented axon filaments. Pt/Pd shadowed. Magnification: 19 000 ×.

Figs. 27, a, b. Fresh transverse frozen sections (1 μ) through the lateral funiculus of frog cervical spinal cord. Myelinated fibres of all calibres (down to 1 μ) are seen. Section kept in Tasaki solution. Phase contrast. Magnification: 600 ×.

Fig. 28. Electron micrograph of transverse fresh frozen section through small (1–2 μ) myelinated fibres from the frog spinal cord. (Same preparate as in Fig. 27 a.) Axon filaments within the opaque myelin sheaths are seen in this 1 μ thick section. Osmium fixation. Pt/Pd shadowed. Magnification: 16 000 ×.

Fig. 29 a. Larger myelinated nerve fibre with straight cylindrical sheath, isolated by microdissection from the fresh lateral funiculus of rat spinal cord. Notice fine myelinated fibre with fusiform enlargement branching off. Tasaki solution. Phase contrast. Magnification: 600 ×.

Fig. 29 b. Small myelinated fibre with fusiform enlargements from lateral funiculus of frog spinal cord. Fresh preparation in Tasaki sol. Phase contrast. 600 ×.

Fig. 29 c. Small myelinated fibre with fusiform swellings from lateral funiculus of rat spinal cord. Notice fine axon filament emerging from broken end. Fresh preparation in Tasaki sol. Phase contrast. 600 ×.

Fig. 29 d. Fine nerve fibre with fusiform enlargements from lateral funiculus of frog spinal cord. Fresh preparation in Tasaki sol. Phase contrast. 1600 ×.

Fig. 29 e. Finest nerve fibres with fusiform enlargements from lateral funiculus of frog spinal cord. These fibres are already close to the limit of visibility and only the enlargements can be made out clearly. Fresh preparation in Tasaki sol. Phase contrast. 1600 ×.

Fig. 29 f. Unmyelinated nerve fibre from lateral funiculus of rat spinal cord. Notice irregular dark bodies adhering to the surface of the fibre. Fresh preparation in Tasaki sol. Phase contrast. 600 ×.

Fig. 29 g. Fine unmyelinated fibre with smooth, wire-like appearance from lateral funiculus of frog spinal cord. Fresh preparation. Phase contrast. 600 ×.

Fig. 30. Submicroscopic nerve fibre of medium size from lateral funiculus of frog spinal cord. A bundle of thin filaments (100 Å diameter) emerges from the broken end. Notice uniform fibre shaft with granular sheath membrane and dense fusiform enlargements. Osmium fixation. Pt shadowed. Magnification: 20 000 ×.

Fig. 31. Submicroscopic nerve fibre of medium size with fusiform enlargement from lateral funiculus of frog spinal cord. Filament bundles flow out from the severed end after running longitudinally through the axon, surrounded by a thin sheath membrane. Notice a dense straight fibre, surrounded by distended membrane, running parallel to the lighter central fibre. Osmium fixation. Pt/Pd shadowed. Magnification: 16 000 ×.

Fig. 32. Submicroscopic nerve fibre of medium size with fusiform enlargements from lateral funiculus of frog spinal cord. Opaque, oval bodies are spaced regularly along the thin sheath membrane which envelops thin fibrous structures. Compare with small fibre running close by. Osmium fixation. Pt shadowed. Magnification: 16 000 ×.

Fig. 33. Submicroscopic nerve fibre of medium size with fusiform enlargements from lateral funiculus of frog spinal cord. Notice the longitudinal strands of fine filaments enclosed within the thin sheath membrane, to which dense oval bodies and numerous light vesicular granules are attached. Osmium fixation. Pt shadowed. Magnification 16 000 ×.

Fig. 34. Submicroscopic nerve fibre of medium size with large fusiform dilatation from lateral funiculus of frog spinal cord. The globular enlargement contains 9 dark bodies together with numerous smaller granules. Notice the thin filaments within the sheath and the varicose bands. The tubular configuration of the sheath membrane is suggested by the form of the broken fibre end. Osmium fixation. Pt shadowed. Magnification: 16 000 ×.

Fig. 35. Submicroscopic nerve fibre of medium size with fusiform enlargements from lateral funiculus of frog spinal cord. The dark oval bodies (0.5–1 μ long) are surrounded by conglomerates of smaller vesicular granules. Notice the large dilatation broken off at one end exhibiting the granular structure of the membrane. Osmium fixation. Pt shadowed. Magnification: 16 000 ×.

Fig. 36. Submicroscopic nerve fibre of medium size with fusiform enlargements from lateral funiculus of frog spinal cord. Notice the longitudinal varicose bands associated with the membrane and their connection with the dense formations at the enlargements. Osmium fixation. Pt shadowed. Negative print. Magnification: 16 000 ×.

Fig. 37. Empty sheath of a submicroscopic nerve fibre with fusiform enlargements from lateral funiculus of frog spinal cord. The tubular character of the single membrane sheath is apparent at the broken end where the outline of the flattened tube walls can be seen. Notice granular fine structure of membrane. Osmium fixation. Pt shadowed. Negative print. Magnification: 16 000 ×.

Fig. 38. Submicroscopic nerve fibre of medium size with fusiform enlargements from lateral funiculus of frog spinal cord. Notice the varicose bands apparently connected with the opaque oval bodies at the enlargements. Osmium fixation. Pt shadowed. Magnification: 16 000 ×.

Fig. 39. Submicroscopic nerve fibre of medium size with dense axonal strand from lateral funiculus of frog spinal cord. The dense strand exhibiting a varicose fibrous structure courses through the lumen of the thin sheath. Notice associated granules of different sizes. Osmium fixation. Pt/Pd shadowed. Magnification: 13 000 ×.

Fig. 40. Fine submicroscopic nerve fibre with fusiform enlargements isolated from lateral funiculus of frog spinal cord. Notice the single, slightly varicose filaments running through the entire length of the fibre, surrounded by a single membrane sheath approximately 1000 Å wide. The internal course of the filament can be clearly seen at the broken edge of the diagonal fibre, where it emerges from within the tubular sheath. Osmium fixation. Pt shadowed. Magnification: 16 000 ×.

Fig. 41. Fine submicroscopic nerve fibre with fusiform enlargements from lateral funiculus of frog spinal cord. The uniform shaft of this fibre has a diameter of only 1000 Å and consists of a single tubular membrane fitting closely around a single filament 200 Å wide. Fairly symmetrical enlargements (0.5 μ) are spaced at regular intervals of ca. 4 μ and contain small opaque bodies or varicose dilatations of the filaments, which course practically unbroken through the entire fibre. Osmium fixation. Pt shadowed. Magnification: 16 000 ×.

Fig. 42. Fine submicroscopic nerve fibre with large fusiform dilatation from lateral funiculus of frog spinal cord. Notice the long thin internal filaments and the varicose bands which seem to be attached to the surface of the sheath membrane in certain places. Osmium fixation. Pt shadowed. Magnification: 16 000 ×.

Fig. 43. Fine submicroscopic nerve fibre with fusiform enlargements of different sizes, isolated from the lateral funiculus of frog spinal cord. A single filament 100–200 Å in diameter can be seen running through the tubular sheath with its irregular enlargements which contain dense granules. Osmium fixation. Pt/Pd shadowed. Magnification: 18 000 ×.

Fig. 44. Club-like ending of a short collateral from a small myelinated nerve fibre of the frog spinal cord. The dense collateral fibre (1000 Å diameter) is invested with a granular sheath and terminates in a circumscript oval body (1 μ long) which contains a few dark granules. Osmium fixation. Pt/Pd shadowed. Magnification: 10 000 ×.

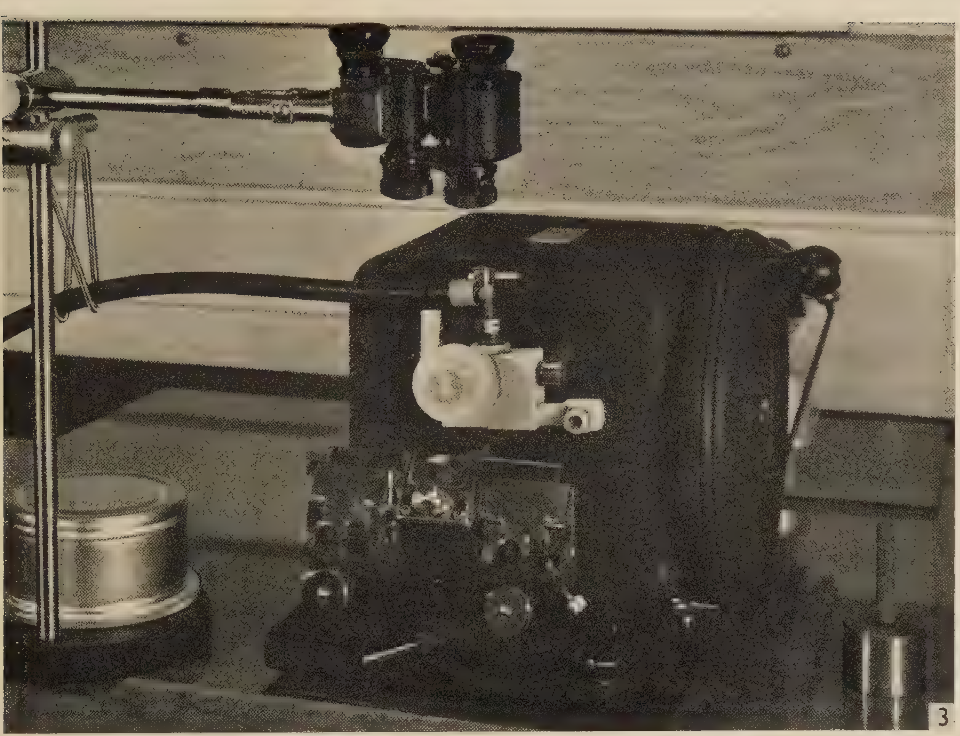
Figs. 45, a, b, c. Terminal formations of fine submicroscopic fibres from the lateral funiculus of frog spinal cord. These sharply outlined globular endings of the finest fibres seem to be enveloped in a membrane and contain several dark bodies (0.5 μ diameter). The thin filament of the fibre often leads into the globular endings. Osmium fixation. Pt shadowed. Magnifications: 16 000 ×.

Fig. 46. Unmyelinated nerve fibre from the lateral funiculus of frog spinal cord. The compact, opaque cord loosens up in certain areas revealing its constituent bundles of tightly packed fine filaments (100–200 Å diameter). Notice the thin (100 Å) sheath membrane which has slipped off from the axon and contains a long, dense nuclear body. Dow Latex calibration particles have been added. Osmium fixation. Pt/Pd shadowed. Magnification: 16 000 ×.

Fig. 47. Unmyelinated nerve fibre from the lateral funiculus of rat spinal cord. The compact axon, which is still visible at the bottom of the micrograph surrounded by its granular sheath, dissociates into numerous thin, nodose filaments (100–200 Å) packed close together in parallel array. Notice remnants of the granular sheath and its oval bodies. Osmium fixation. Pt shadowed. Negative print. Magnification: 16 000 ×.

Fig. 48. Unmyelinated nerve fibre from the lateral funiculus of rat spinal cord. The flattened axon appears here as a "naked" bundle of thin varicose filaments, running longitudinally in a compact arrangement. Notice the nodular appearance of the individual filaments resulting from fairly regular axial discontinuities. Traces of the granular membrane can be discerned. Osmium fixation. Pt shadowed. Negative print. Magnification: 20 000 ×.

Fig. 49. Fine unmyelinated nerve fibre from the lateral funiculus of frog spinal cord. The fibre, which is partly invested with a thin granular sheath, separates into bundles of thin nodular filaments (200 Å diameter) running long independent courses. Notice remnants of the oval bodies associated with the sheath. Osmium fixation. Pt shadowed. Negative print. Magnification: 20 000 ×.



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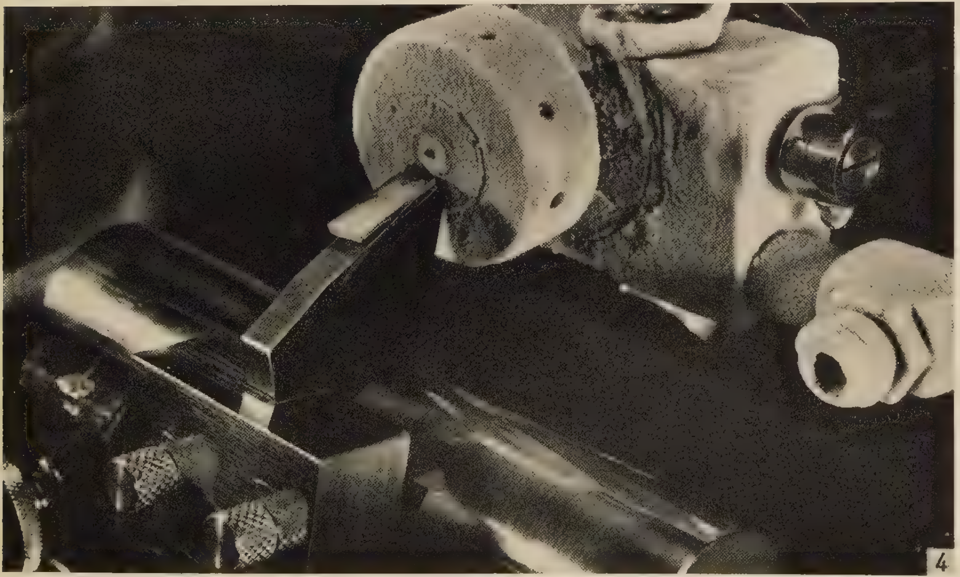

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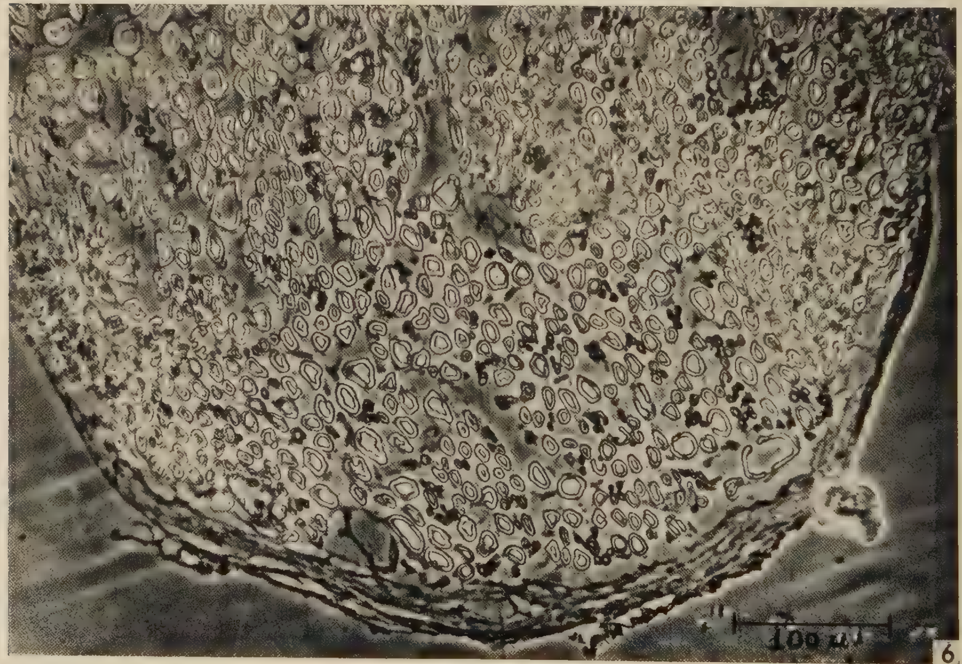

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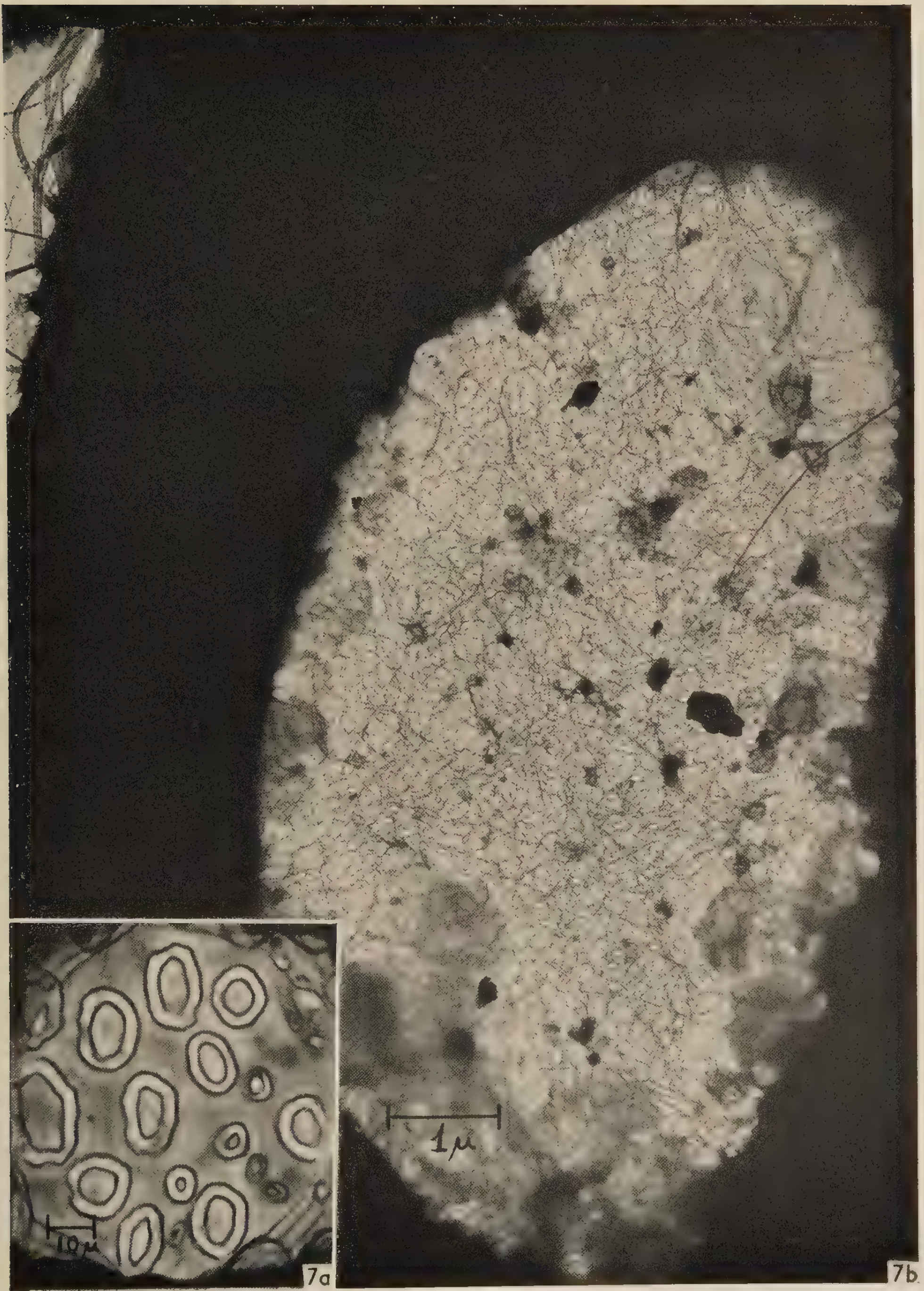
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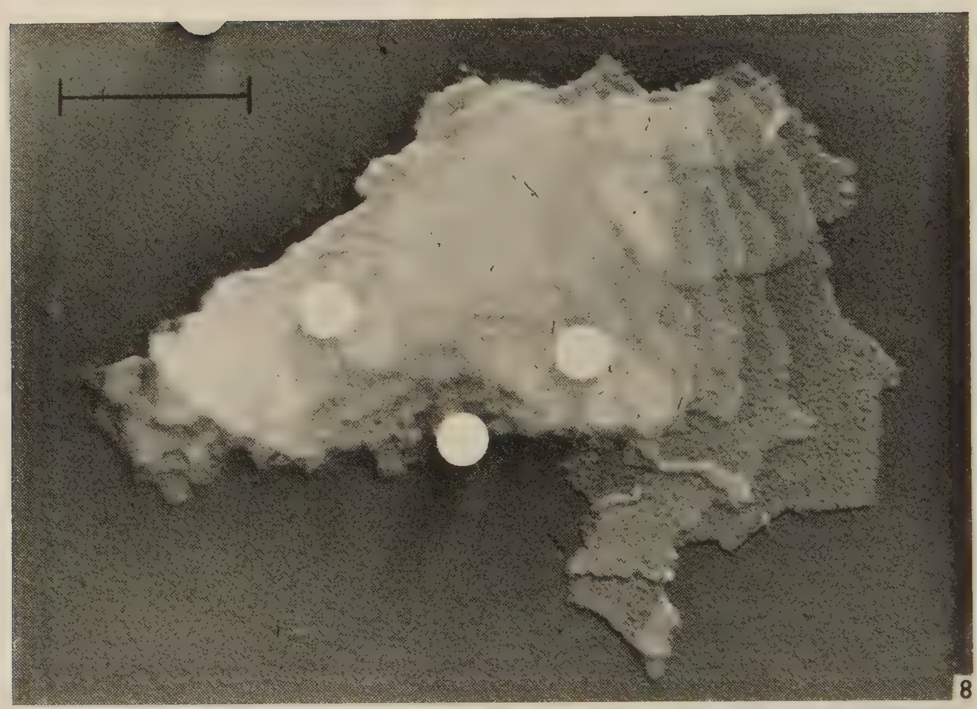

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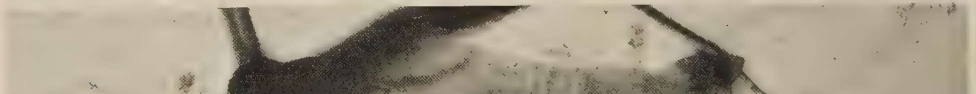

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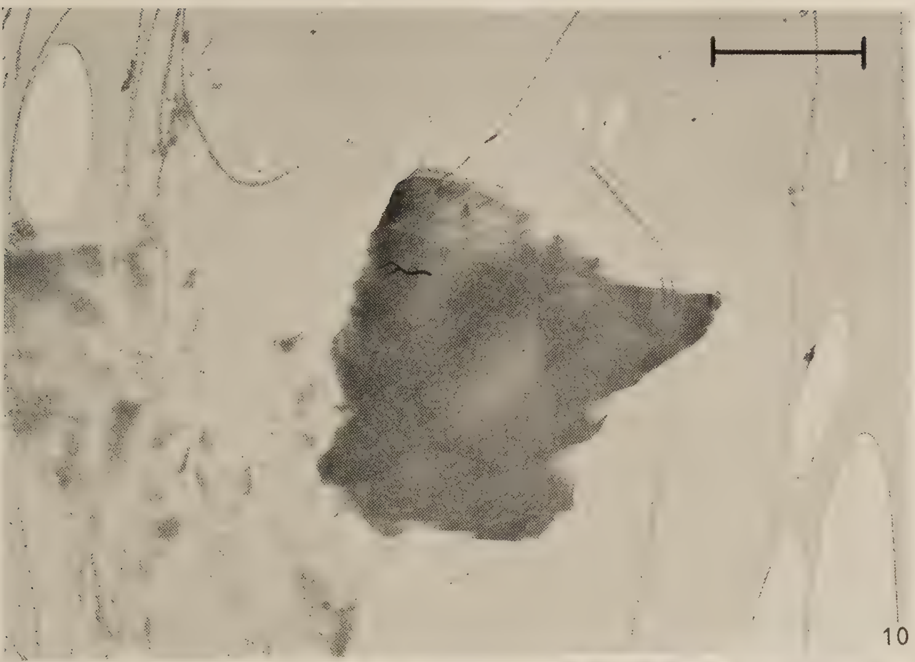
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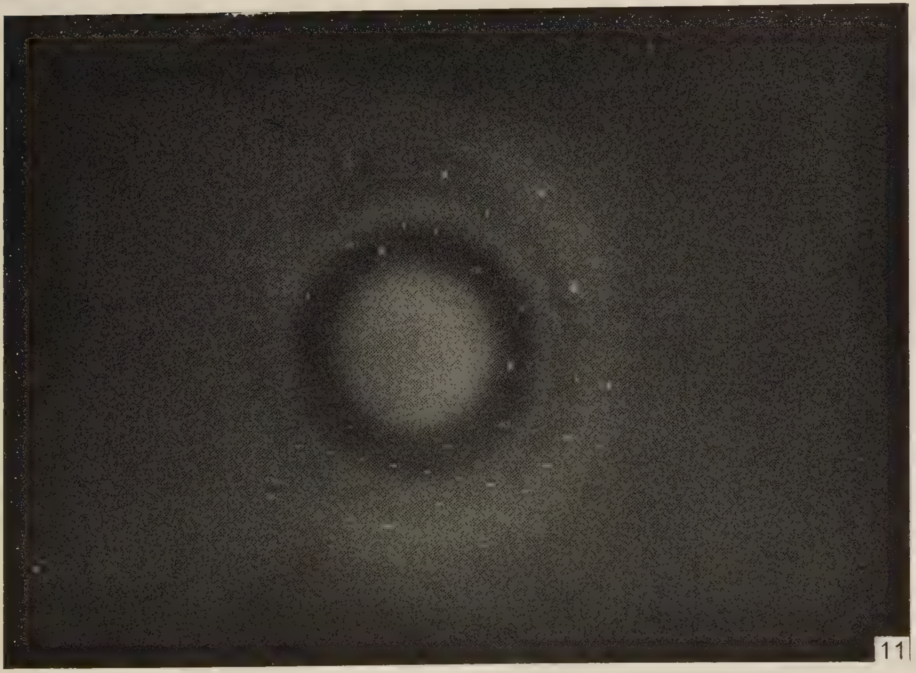
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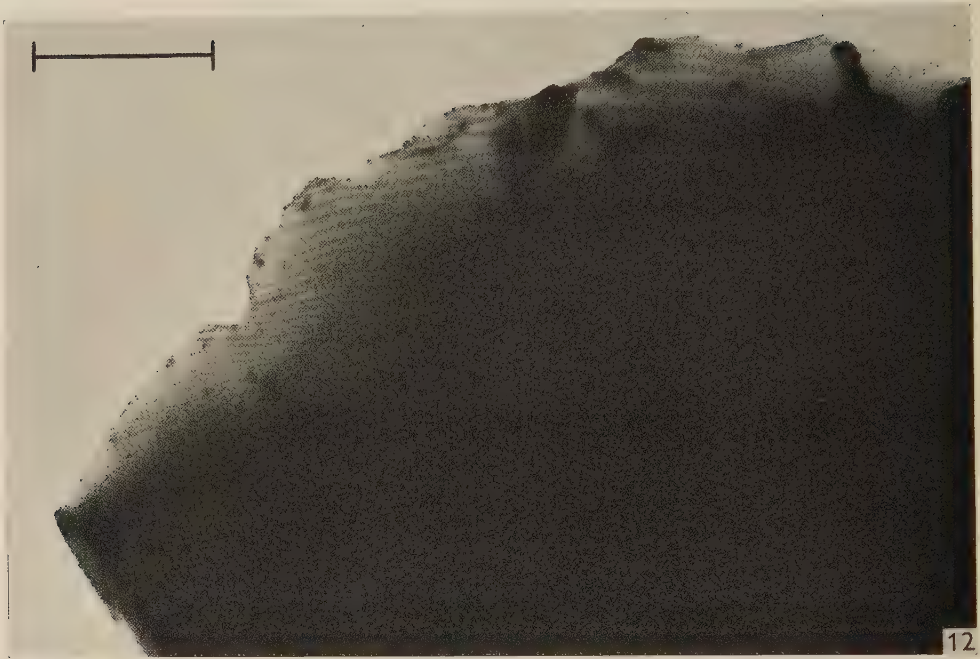

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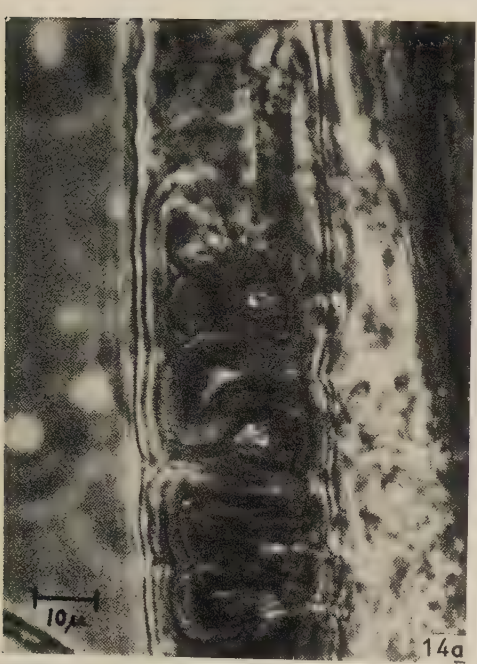

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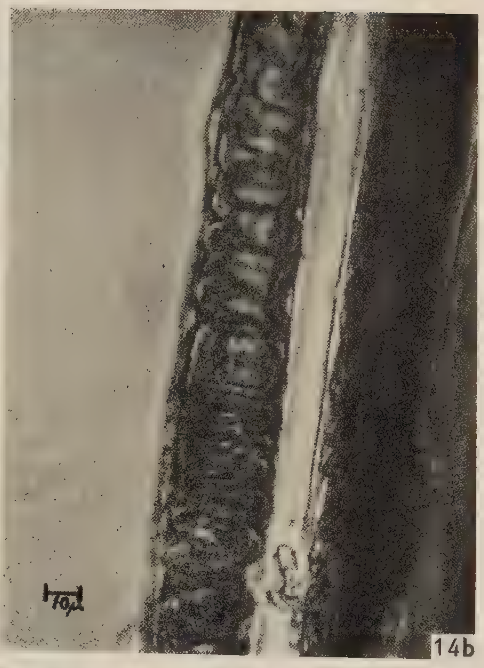

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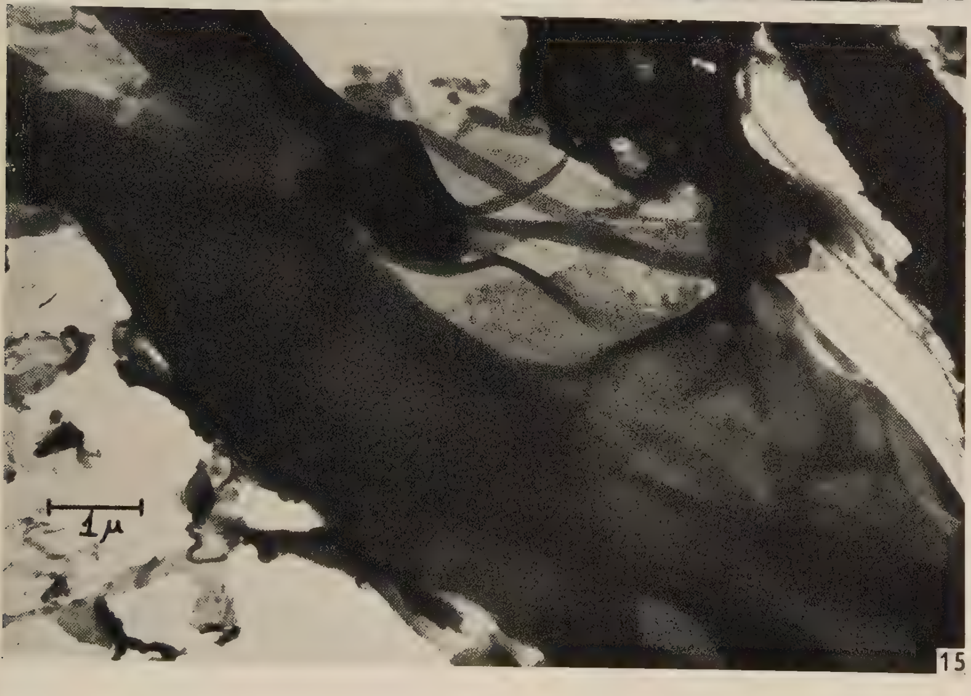

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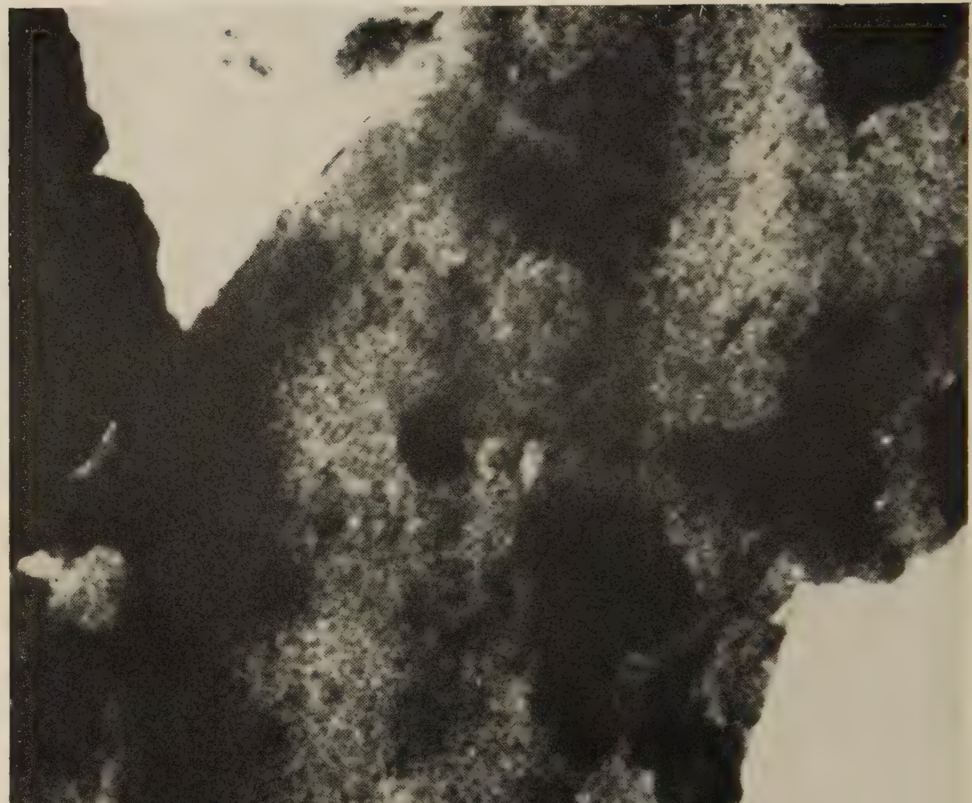

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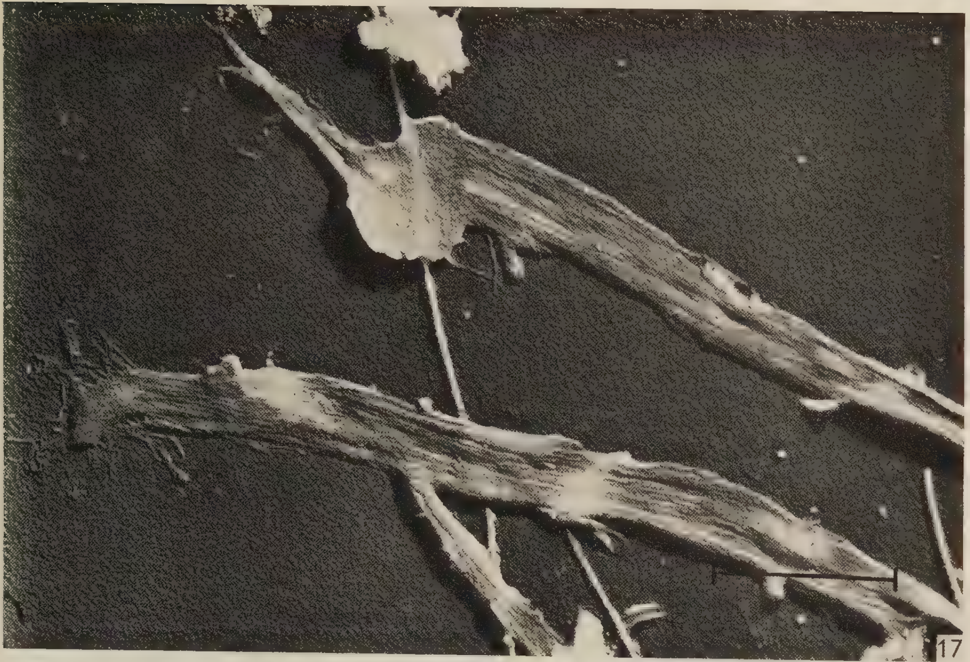

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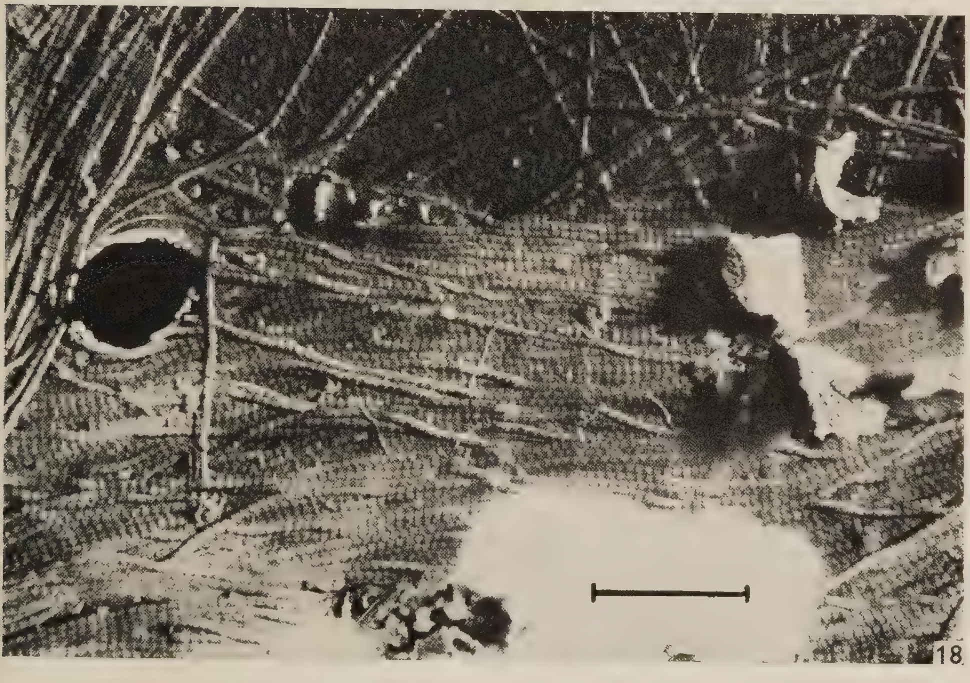

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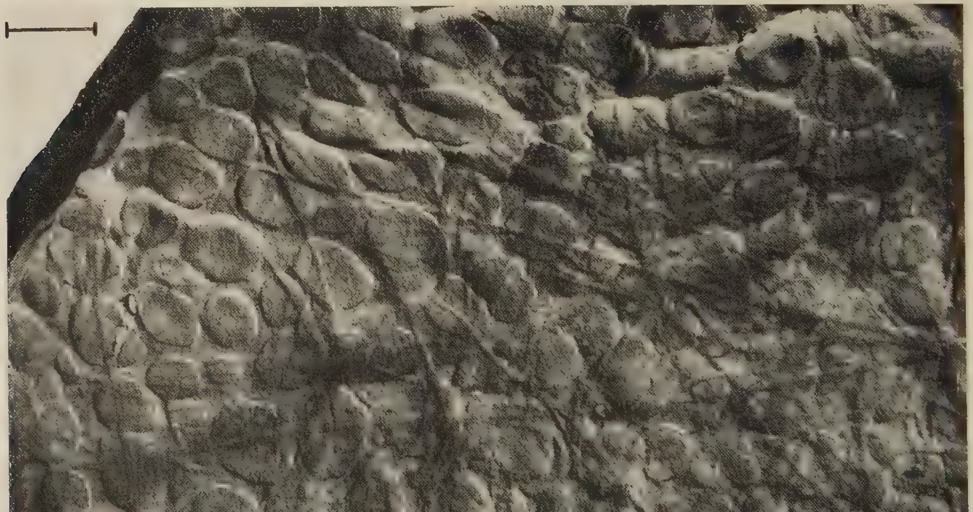

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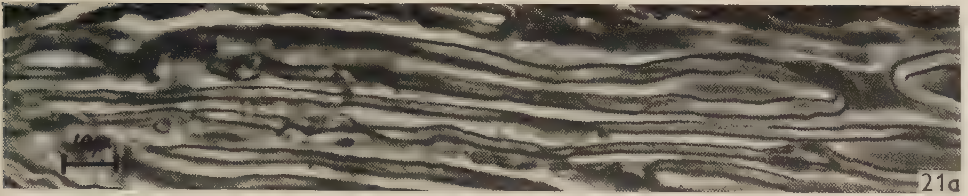

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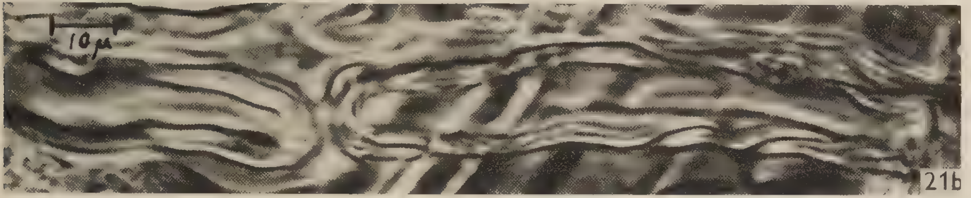

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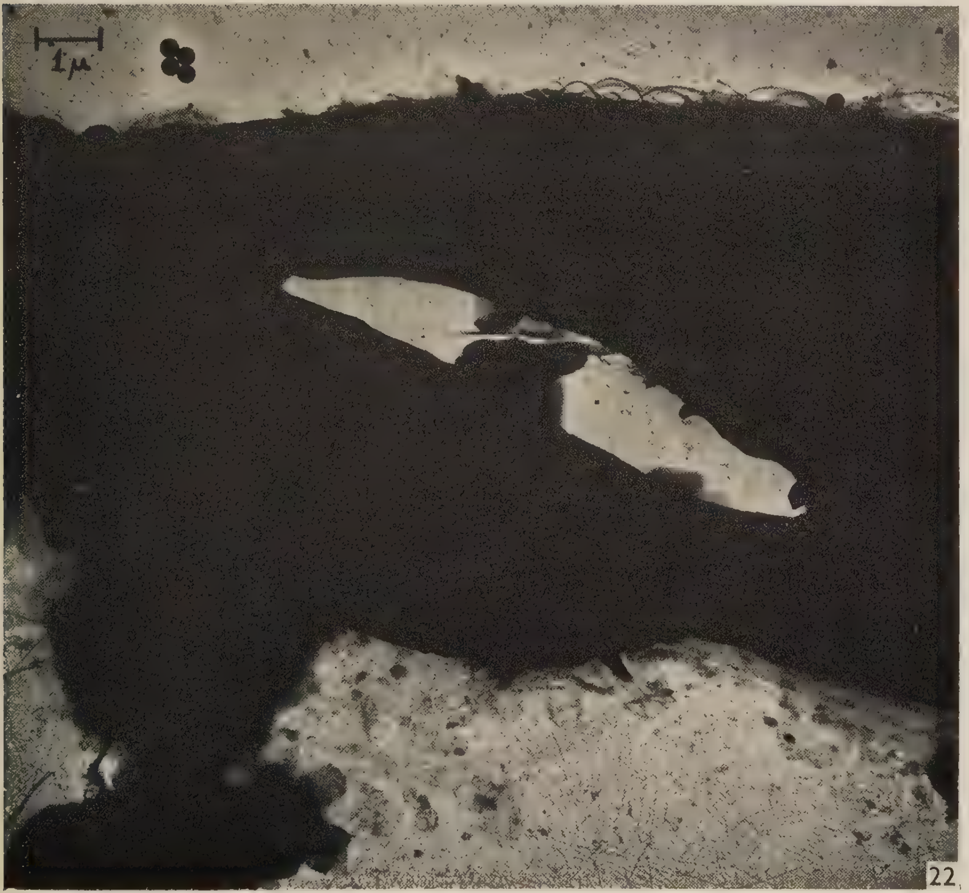

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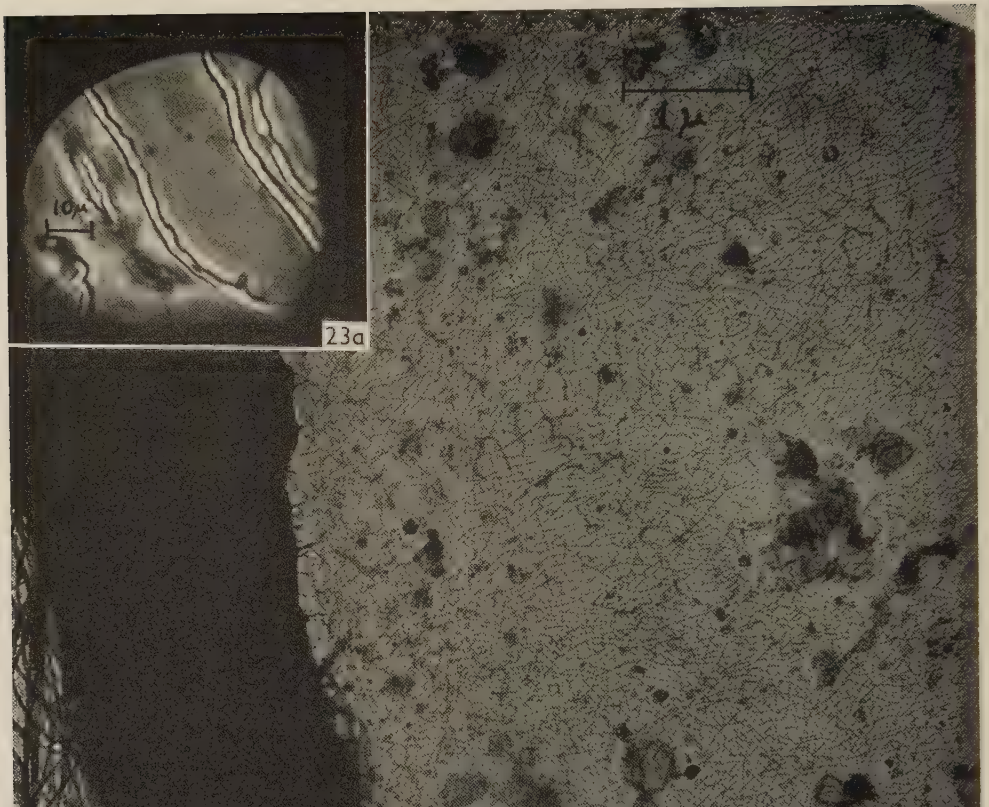

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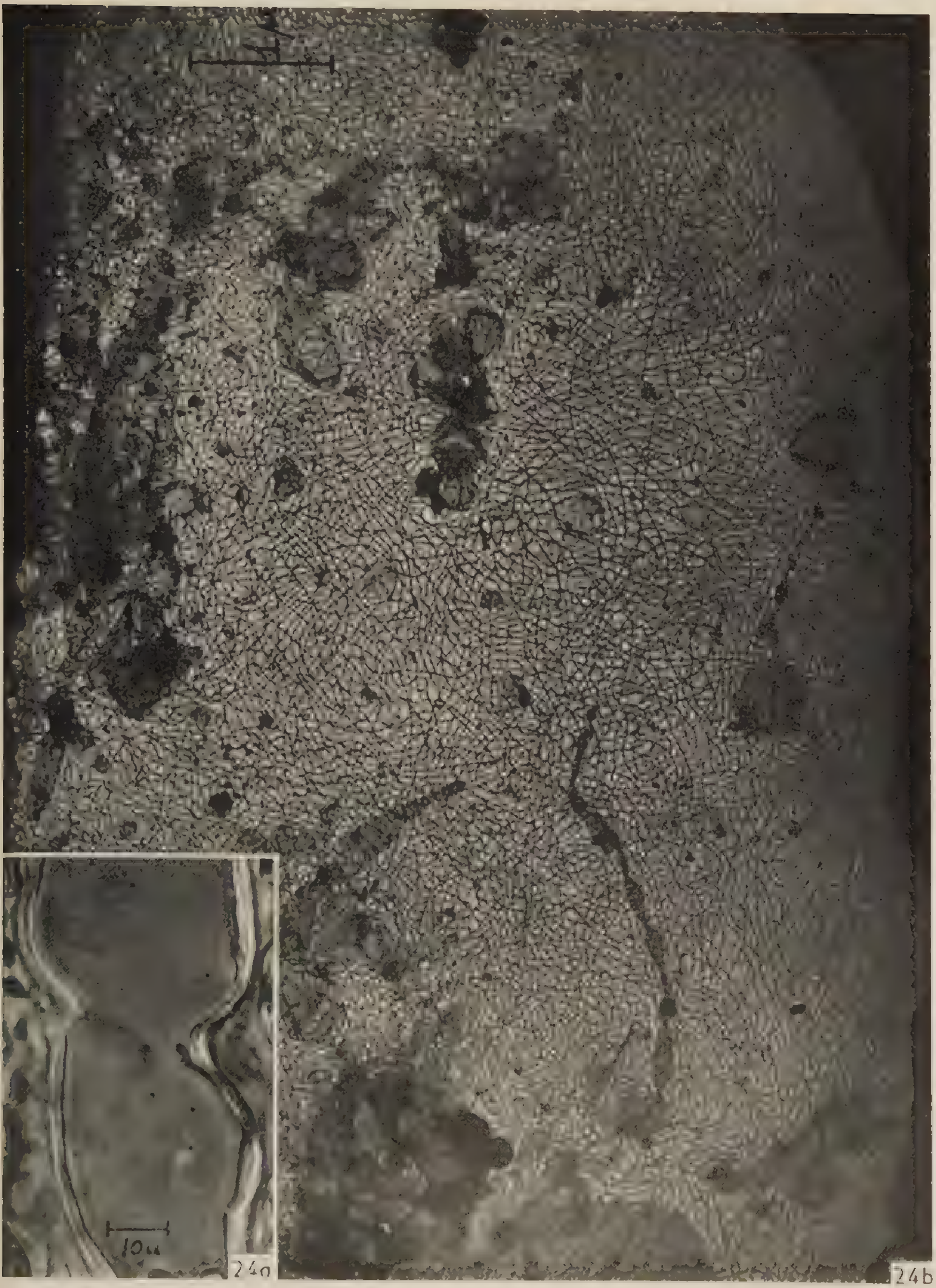
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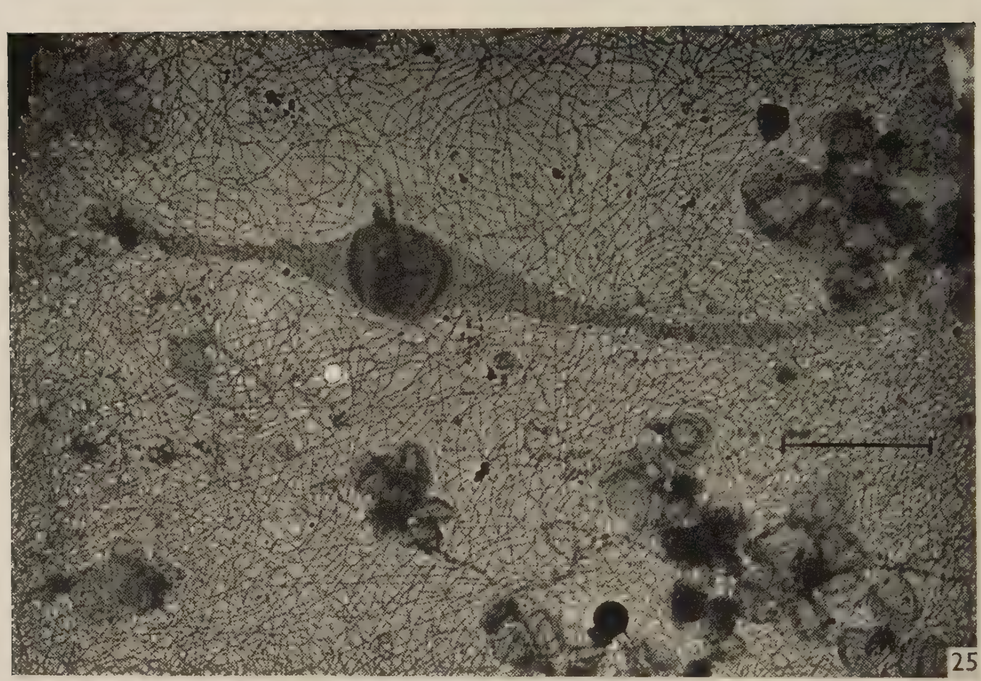
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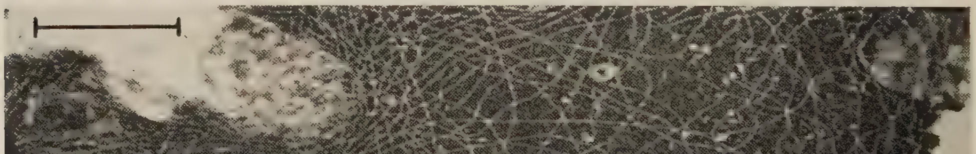
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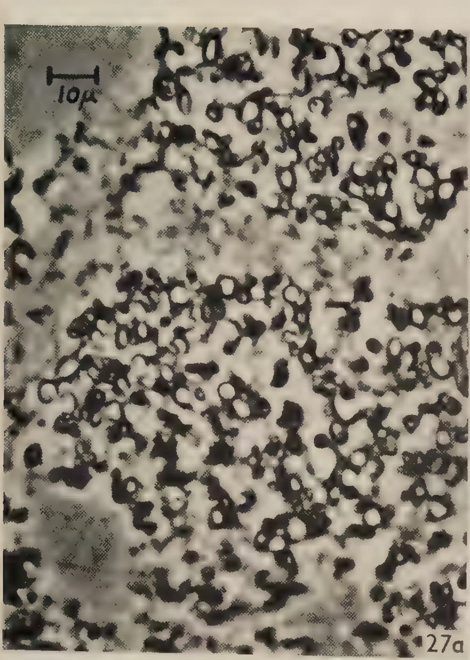

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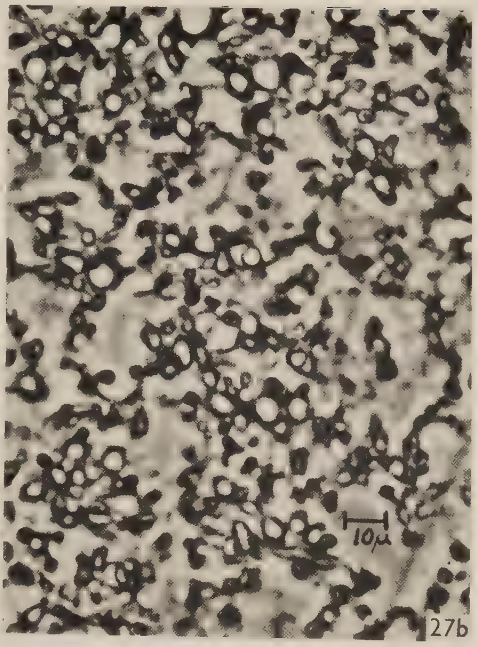

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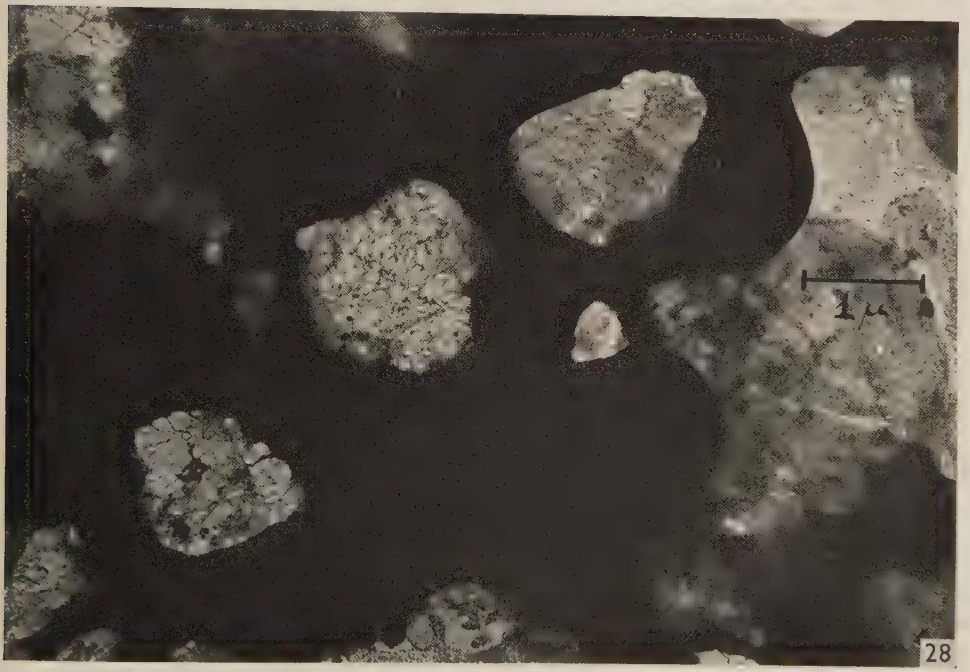

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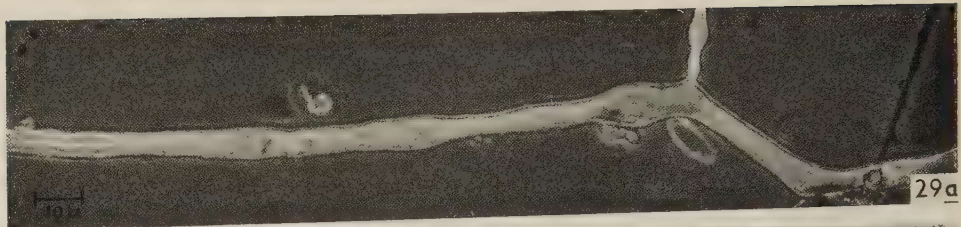

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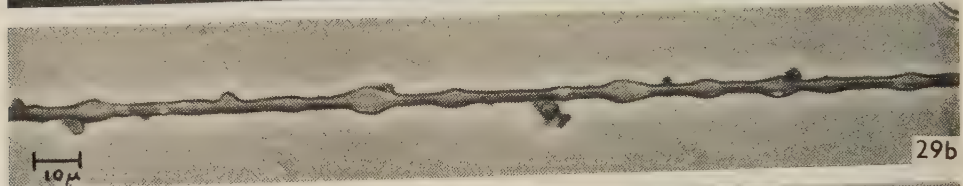

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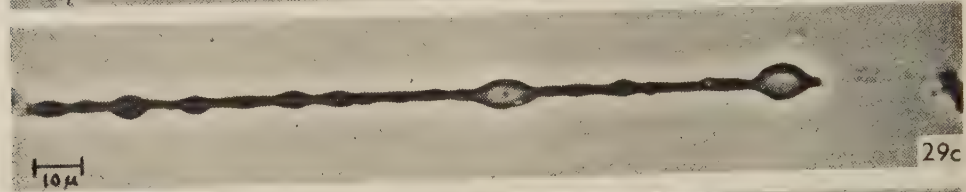

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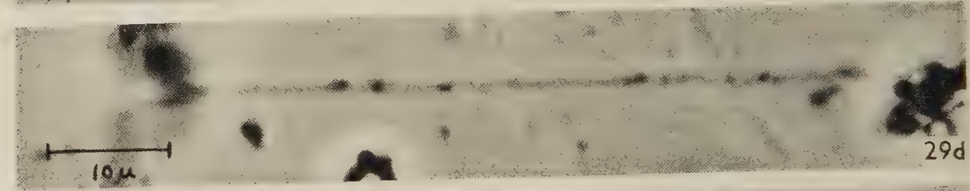

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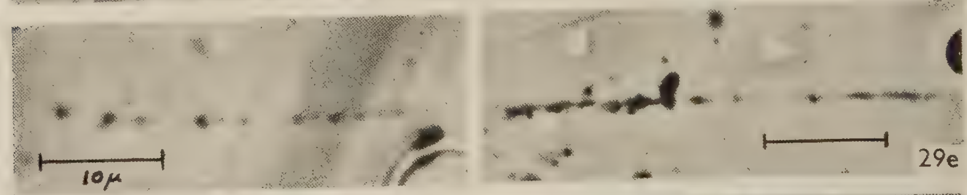

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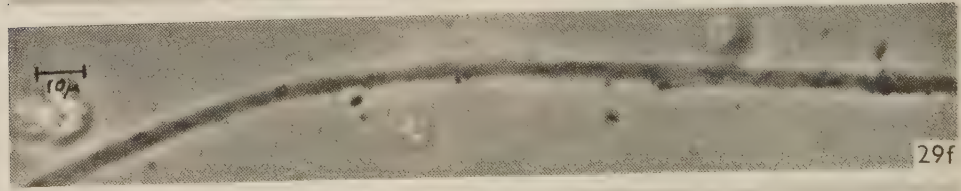

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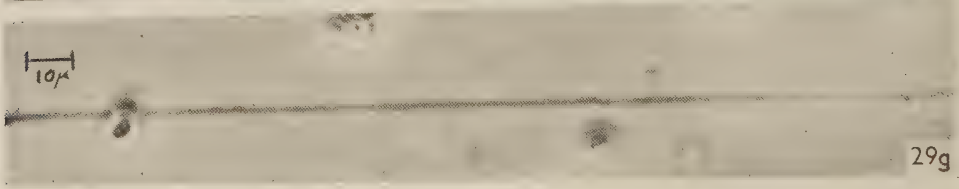

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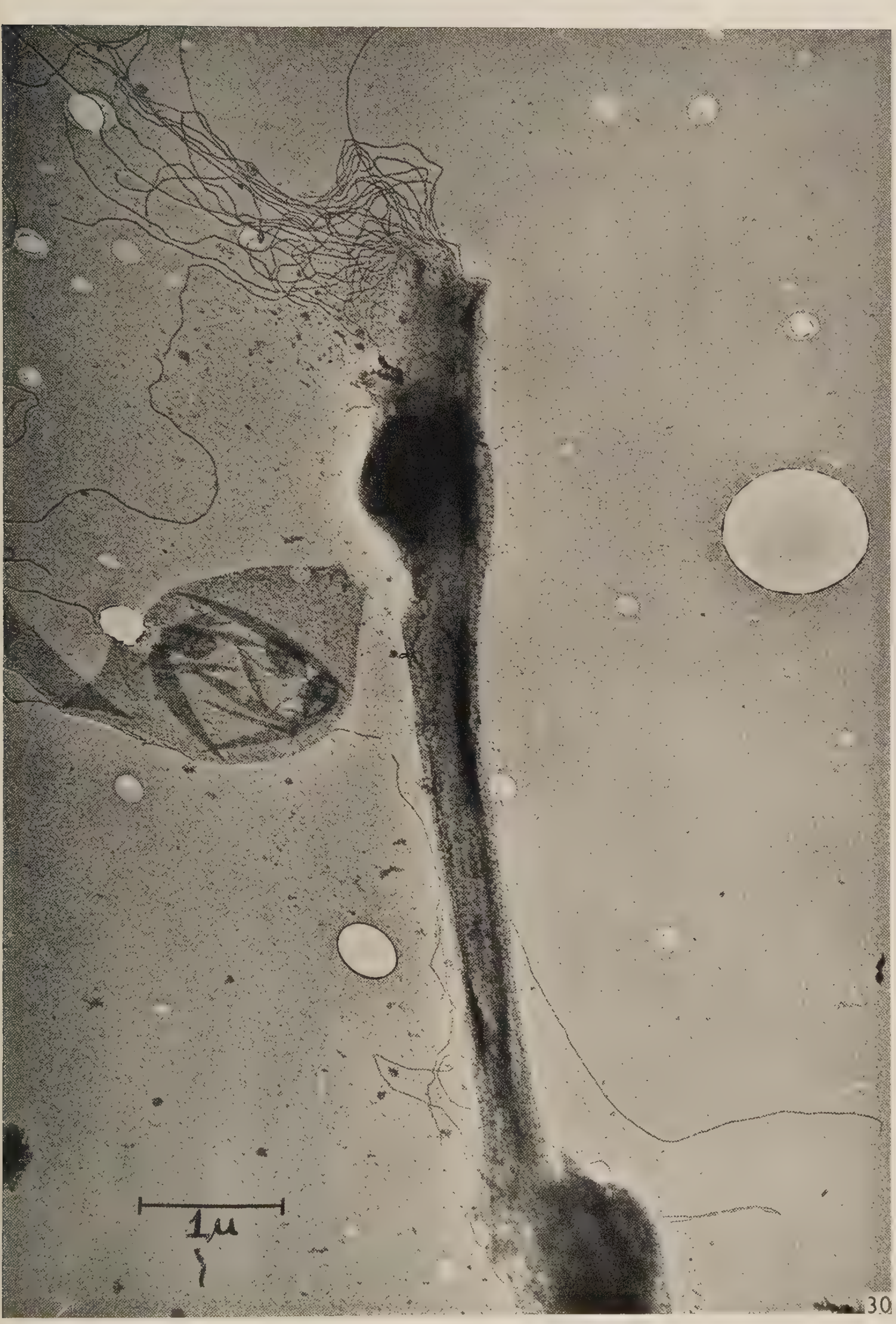

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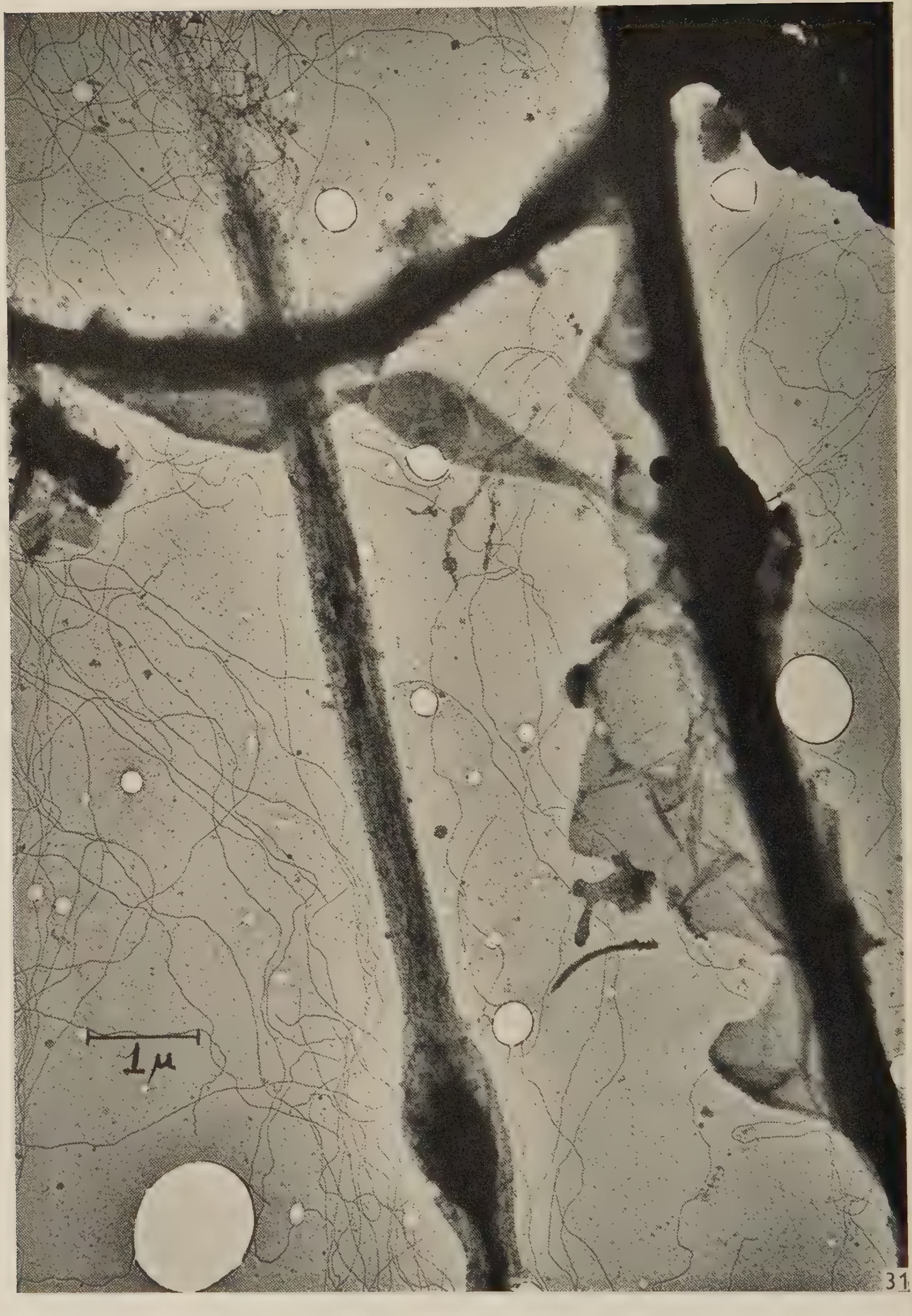
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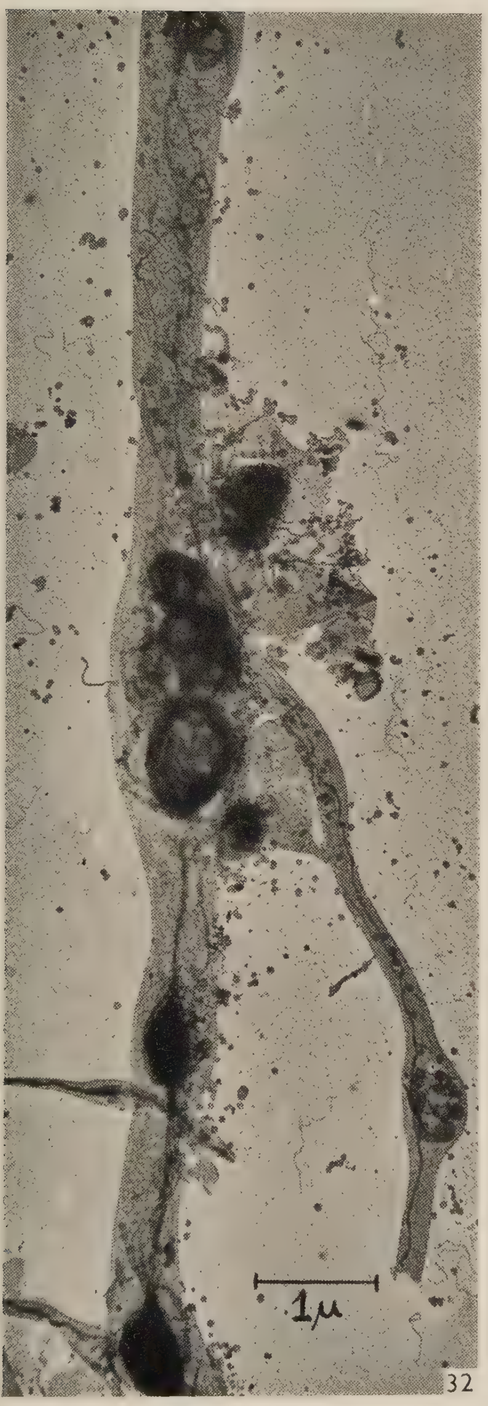

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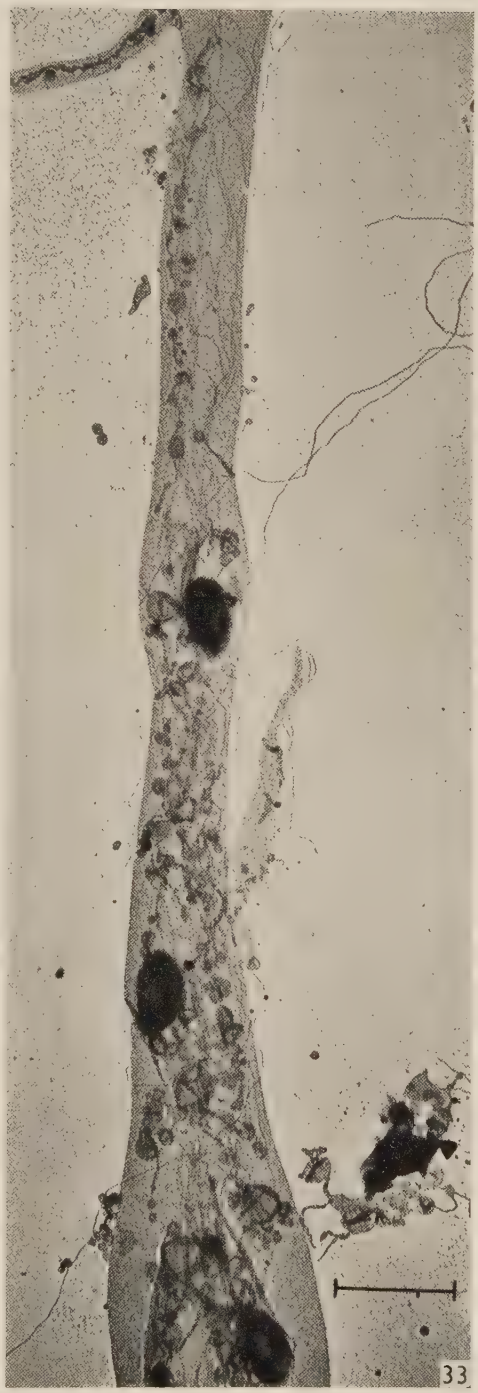

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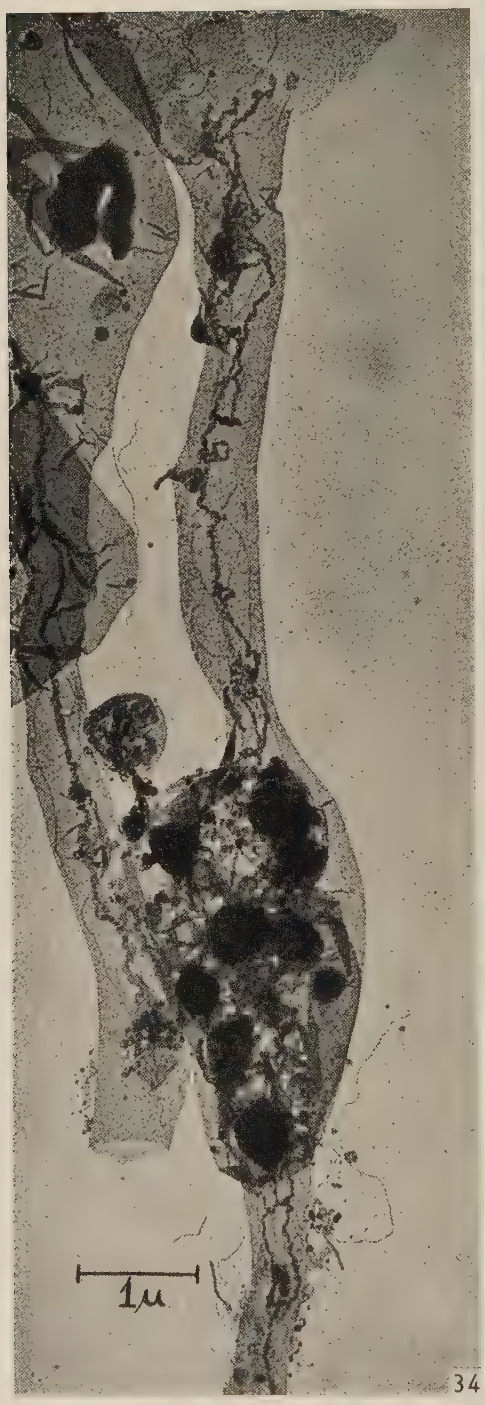

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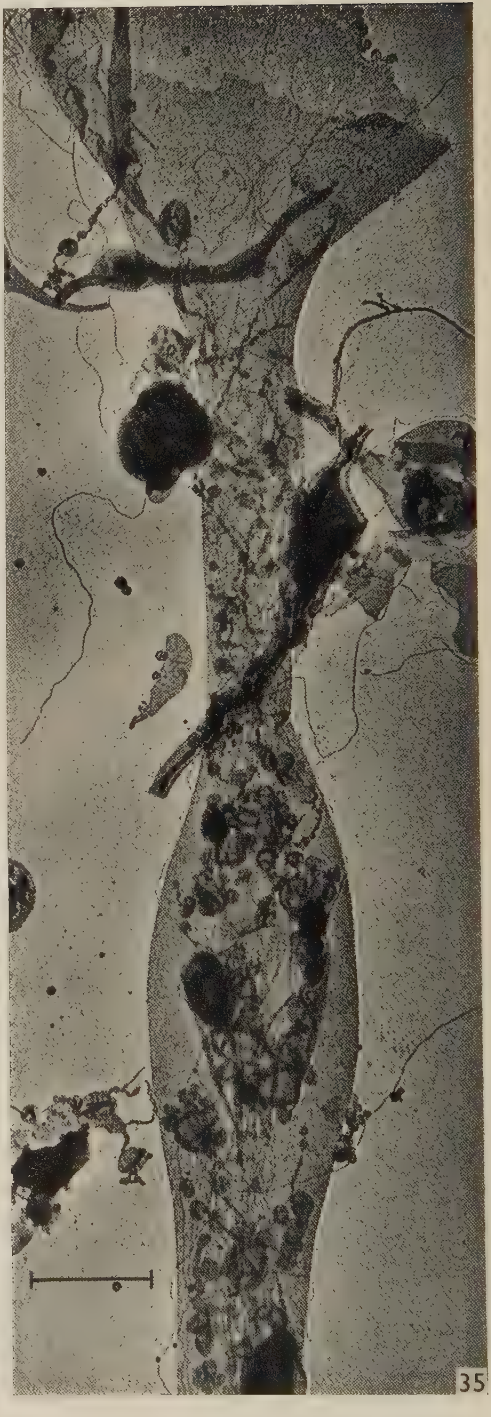

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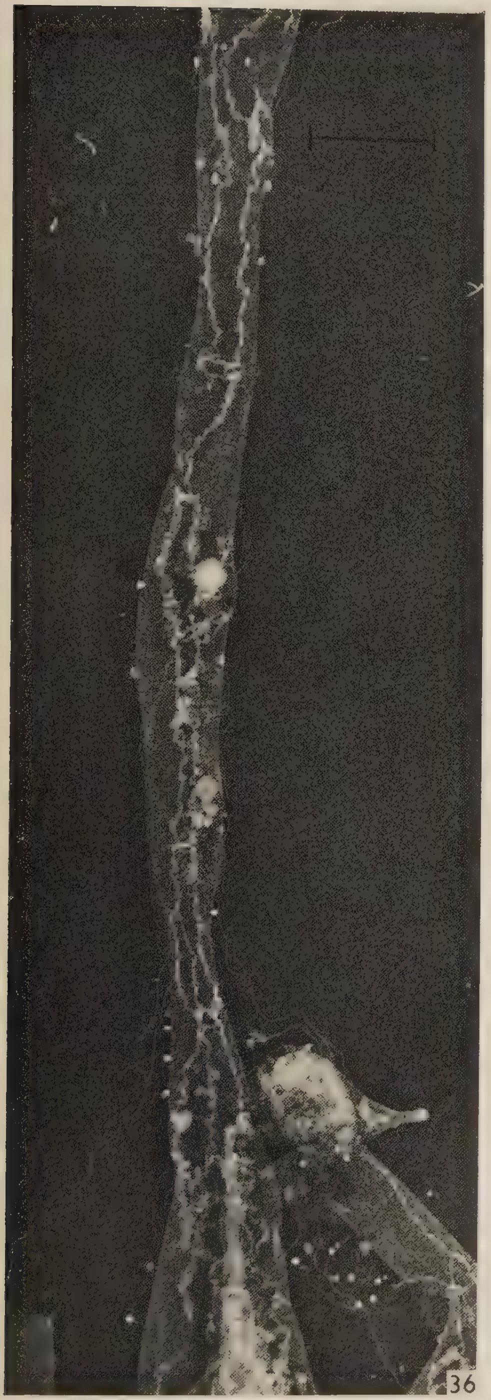

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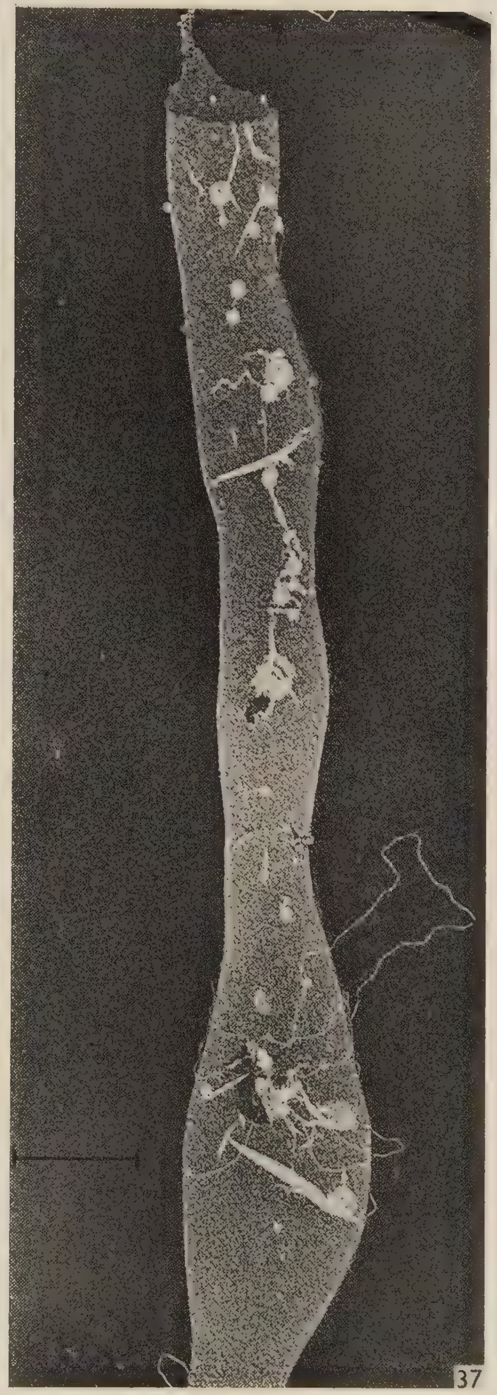

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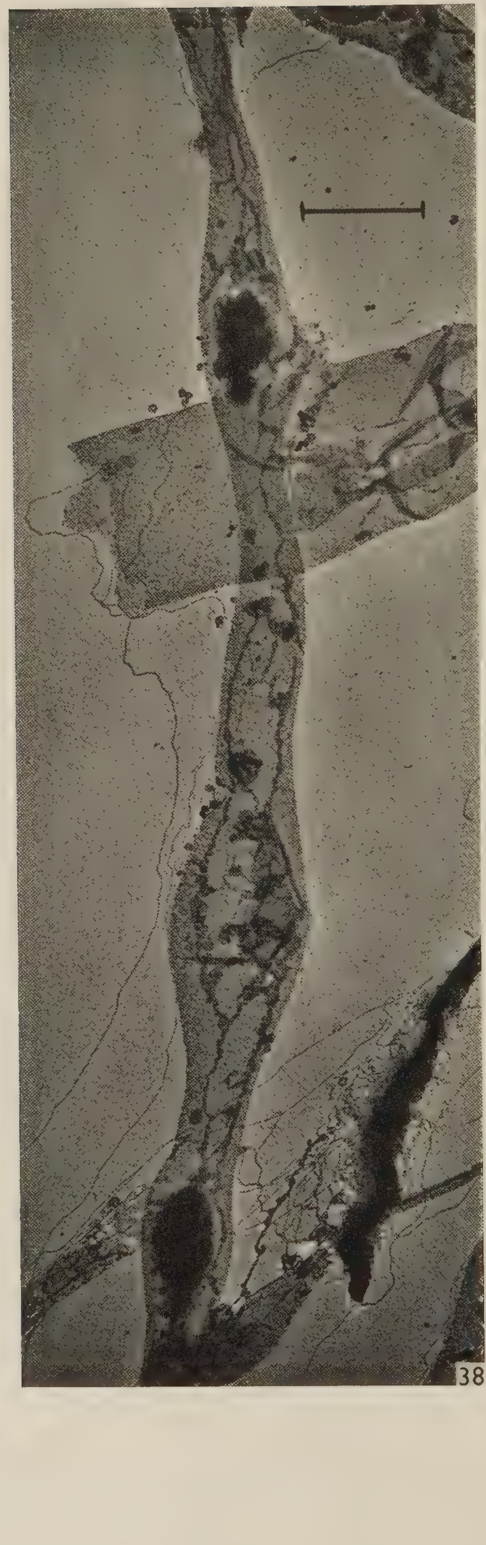
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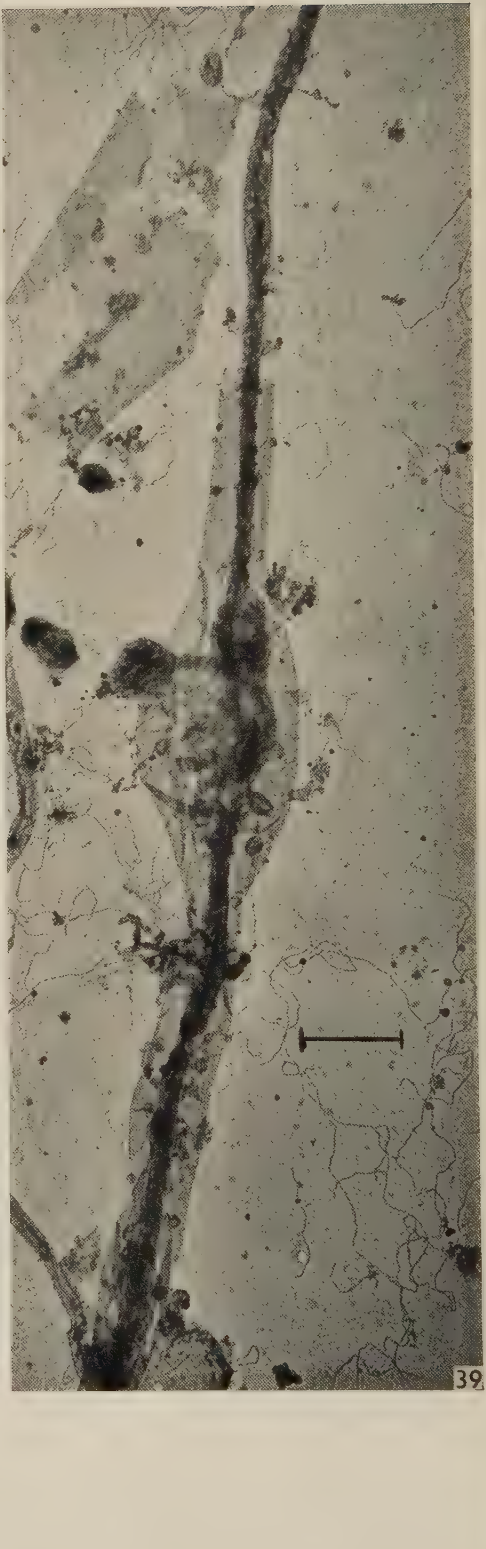
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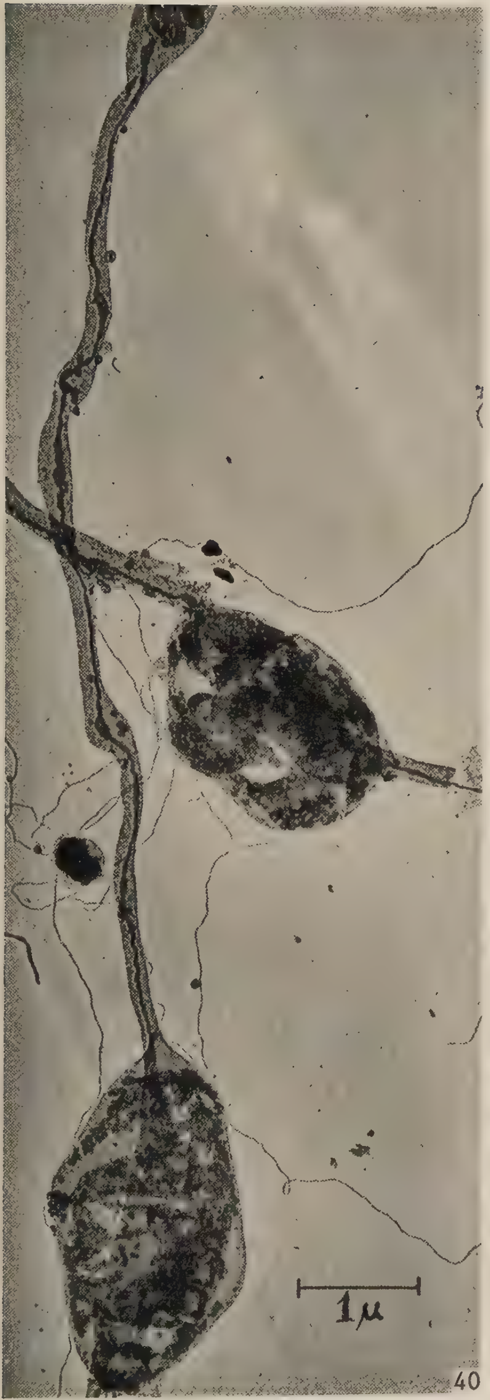

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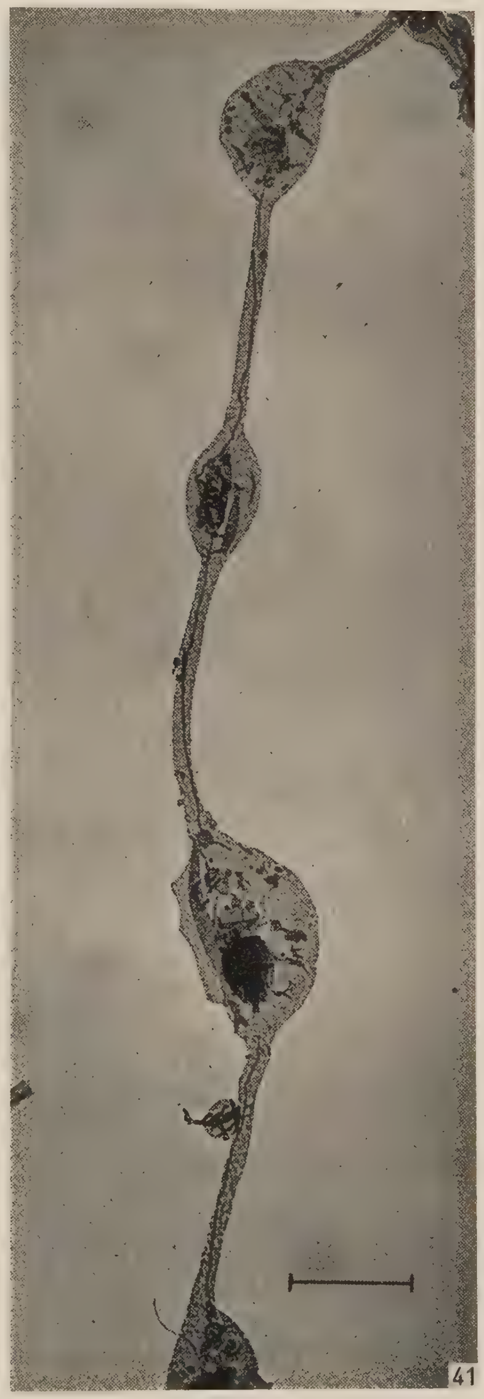

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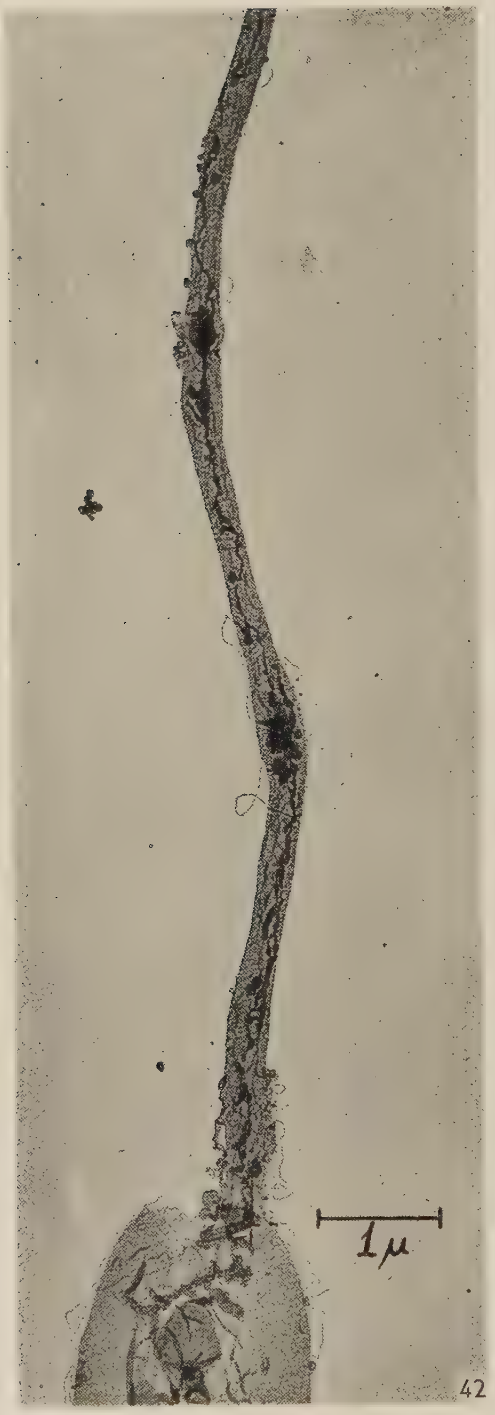
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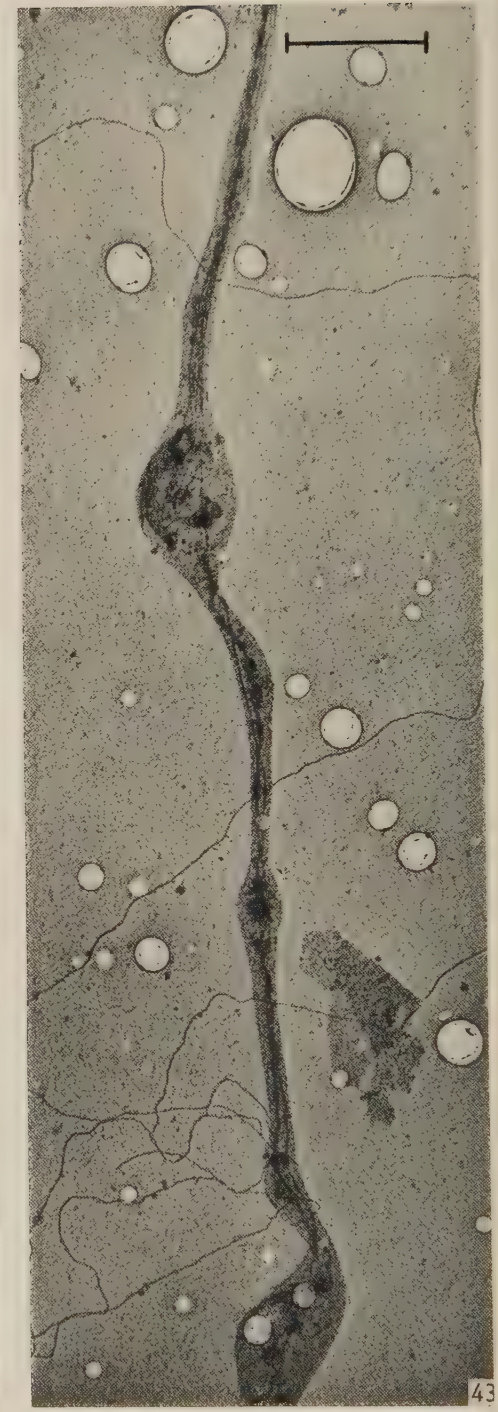
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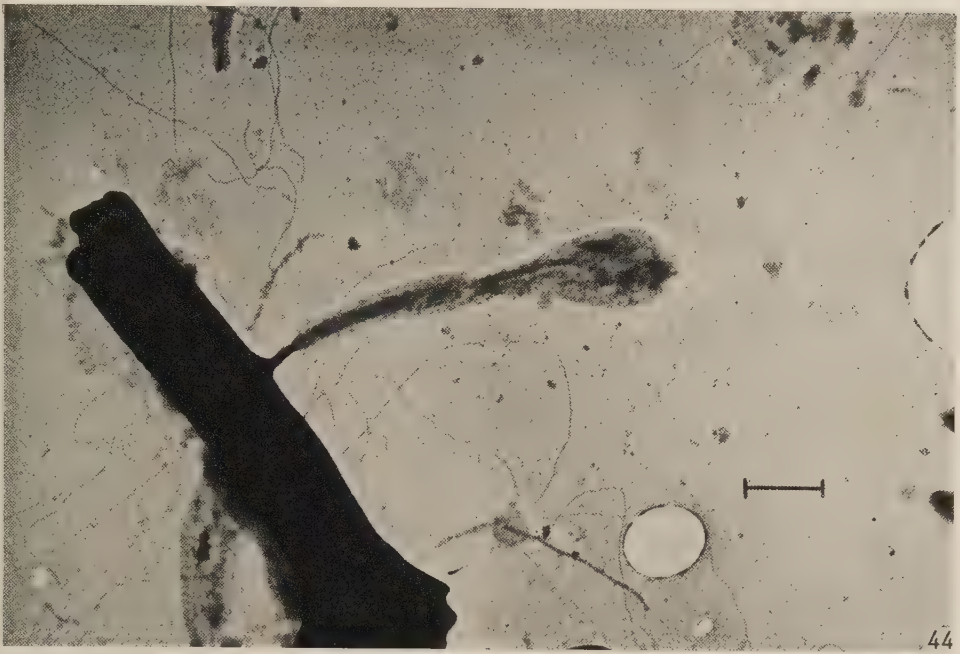
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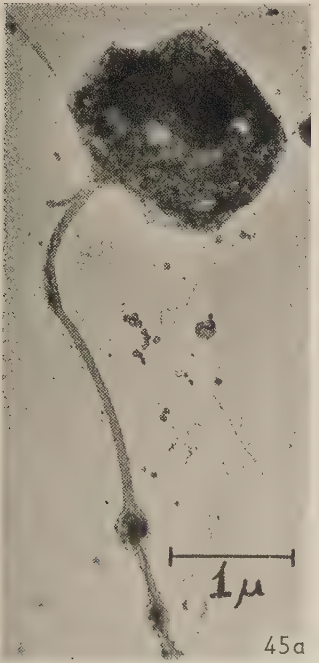

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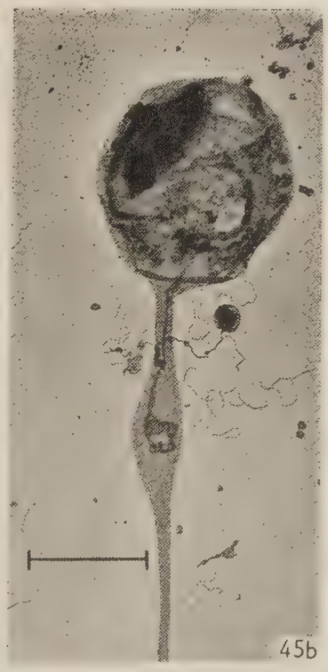
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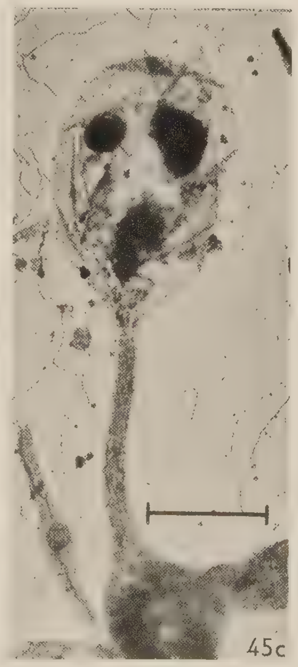
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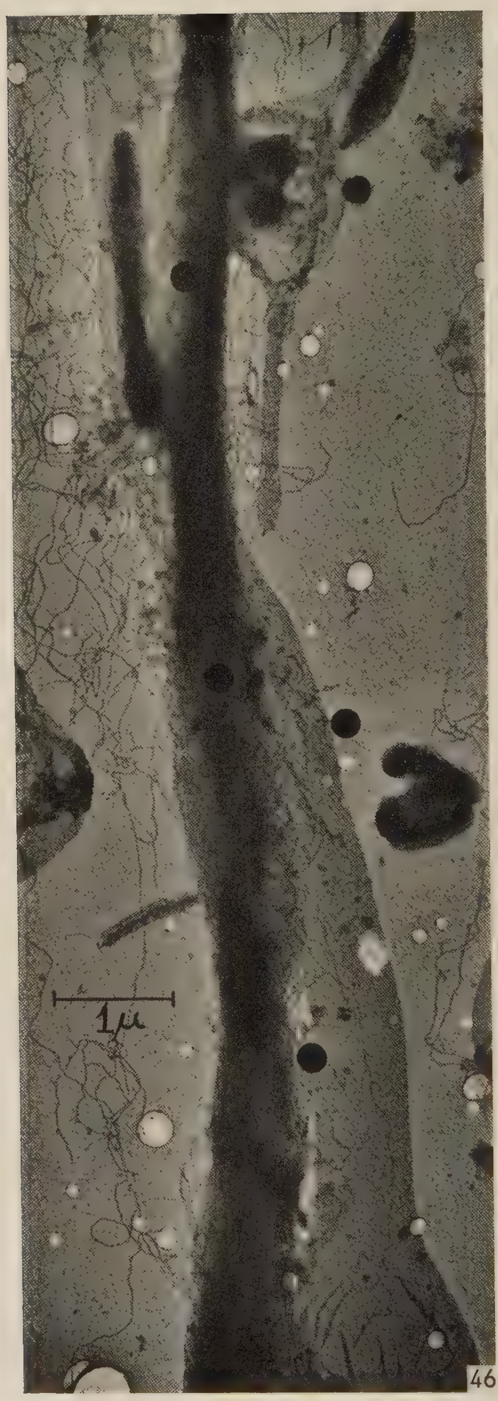

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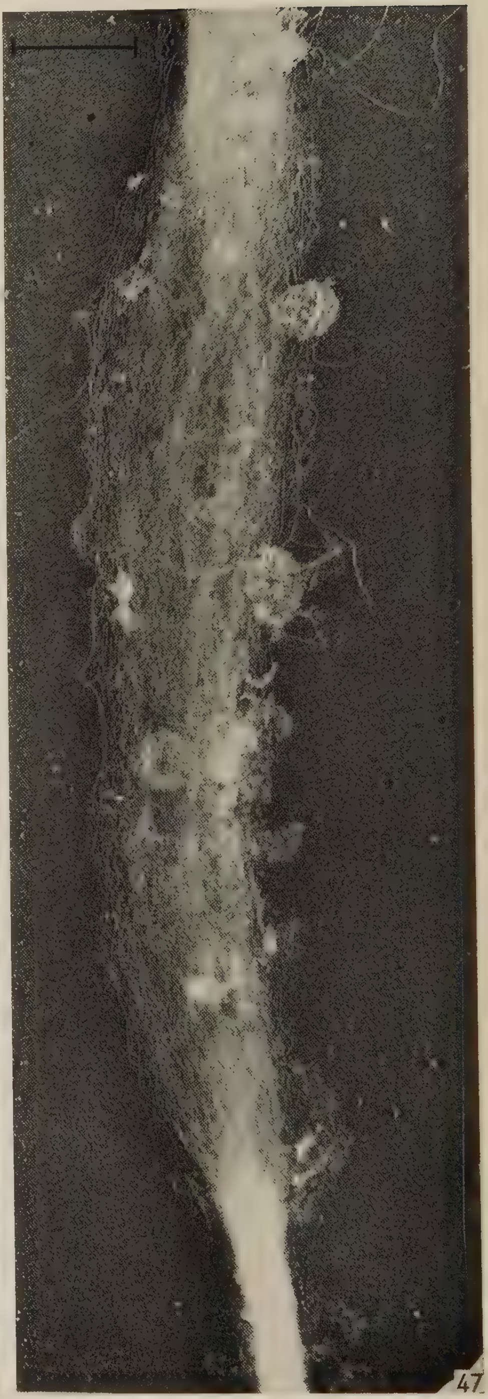

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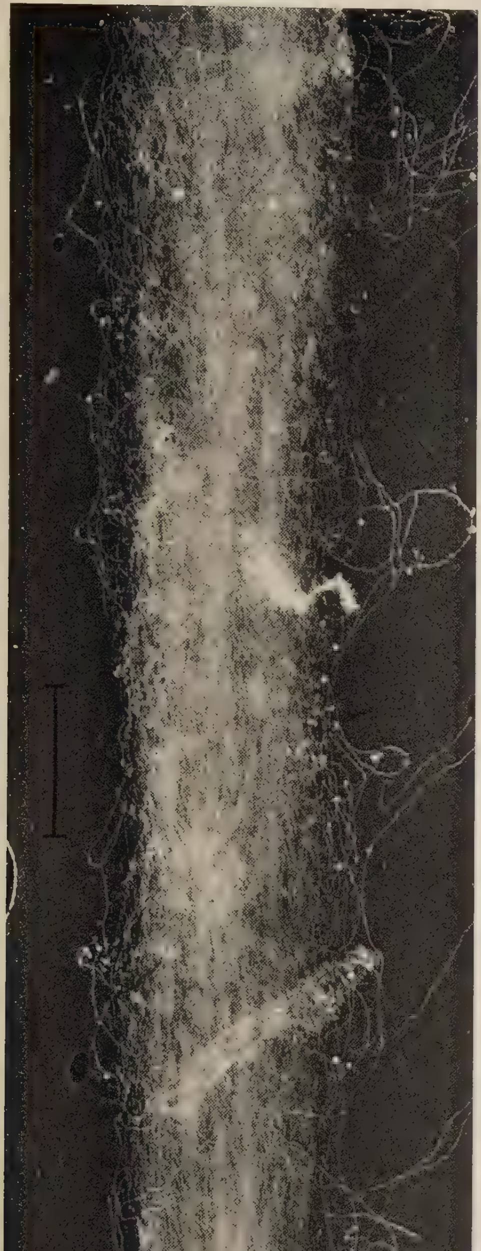

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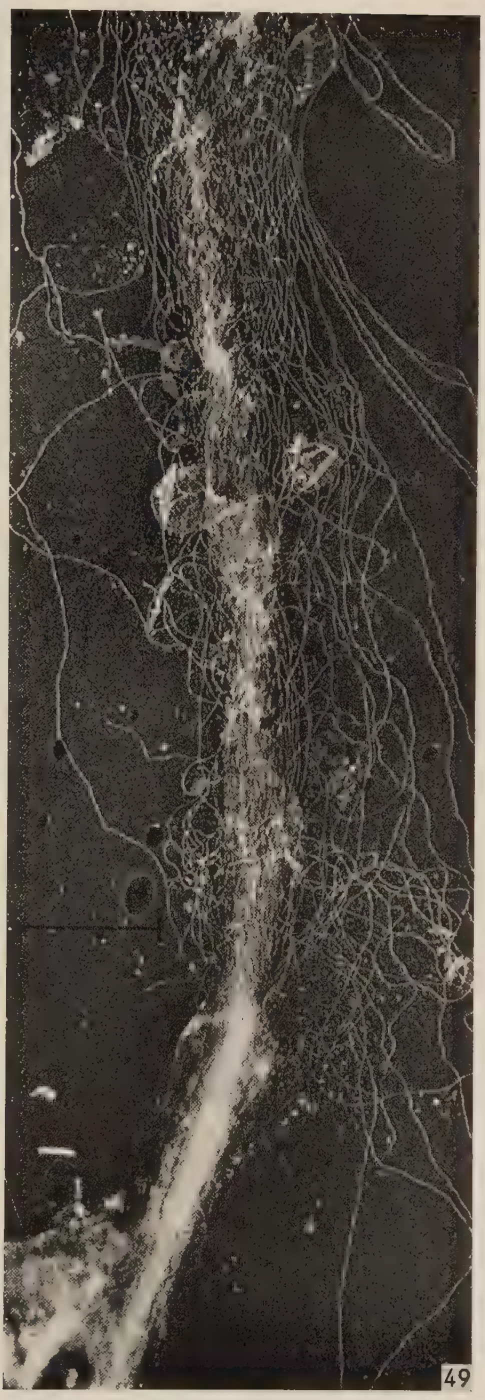

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NUCLEAR ACTIVITIES OF HUMAN FETAL BRAIN CELLS IN TISSUE CULTURES

MARY JANE HOGUE

Department of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

Received July 12, 1951

The study of human fetal brain cells in tissue cultures (2) has brought out the fact that they have a wide variety of nuclear activities. These activities have been seen in brain cells living under conditions as nearly normal as possible. The present paper is a study of three of these nuclear activities, with special attention being given to budding and the formation of multinucleate nerve cells.

MATERIAL AND METHOD

The cells used in these studies came from the brains of human fetuses which varied in length from 22 mm crown rump (C.R.) to stillborn infants at term and also from one infant which lived for one day. The general term "brain cell" is used, as in many cases it was difficult to distinguish some of the small neurons from glia cells. The parts of the brain used for the tissue cultures were the cerebral hemispheres, cerebellum, medulla and pineal body.

Nearly all the living cells used for these studies were full of granules; in such cells the nuclei could be more clearly seen than in the other almost transparent cells. Neutral red was added to a few cultures and helped to define the nucleus by coloring the granules surrounding it.

The parts of the brain to be used for culturing were cut into small pieces, about 1 mm in diameter. These were grown in hanging drop and roller tube cultures. They were fastened to the coverslip or to the walls of test tubes by chicken plasma coagulated with chick embryo extract. The nutrient medium which was added to all cultures was a solution of 6 ml of Tyrode solution, 6 ml of chick embryo extract, and about 6 drops of human placental serum. This solution was changed every 2 to 3 days. The cultures were incubated at 37° C.

In addition to the study of living cells, some of the hanging drop cultures were fixed in Heidenhain's susa solution, stained with Heidenhain's iron haematoxylin and counterstained with fuchsin which demonstrated very distinctly the long fine cell processes.

OBSERVATIONS

The nuclear activities of the brain cells discussed in the present paper are 1) the pouring out of material from the nucleus into the cytoplasm, 2) the formation of small globules, and 3) budding.

In the first of these activities, there appears at one or more points on the surface of the nucleus (fig. 1 A) material pouring out in masses from the nucleus into the cytoplasm. In the same figure, in another cell at B, are clear channels arranged like rays opening out from the nucleus and surrounded by granules. These channels have been observed in cells whose cytoplasm is full of unstained granules and in others where the cytoplasmic granules have been stained with neutral red. The channels are always straight, as though the material they contained was moving out from the nucleus under pressure. Similar channels have also been seen and described (3) in choroid plexus cells.

The second nuclear activity studied is the formation of small globules at the surface of the nucleus. These are pinched off into the cytoplasm. Sometimes a row of these small globules can be seen in a single cell (fig. 4). Occasionally the globules elongate as though they are being pressed upon by the granules surrounding them.

The third nuclear activity, and by far the most spectacular one, is budding. This was observed in both living and fixed cultures. The buds are larger than the globules just described and are of many different sizes. One, two or three buds may form on the surface of the same nucleus. Some are nearly half the size of the nucleus. Figs. 2 and 3 are different stages in bud formation and fig. 9 shows a fixed and stained cell with two buds of slightly different sizes formed on the same nucleus.

In some cultures more nuclear buds are formed than in others. One particular culture had a large number of them. It was from the medulla of an 88 mm C.R. fetus which had been kept at 6° C for 5 days (4) before culturing. However, the cells seemed to be in good condition. Cells with lobed nuclei and binucleate cells were observed from the 23rd to the 39th day of culturing. The culture lived for 46 days.

The large buds often look like lobes of the nucleus, especially in the living cells where it is frequently impossible to say whether the nucleus is lobed or whether two or more nuclei are present (2). Fig. 5, a living cell, has three distinct nuclei present, but in fig. 6, also a living cell, it is difficult to decide whether the nucleus is lobed or whether several distinct nuclei have formed.

The actual separation from the nucleus of these large buds or lobes has not been observed in the living material. However, in fixed and stained preparations many of the larger cells, some of them Purkinje cells (figs. 11, 12), have two or more distinct nuclei (fig. 10). The neuron in figure 8 has seven nuclei and fig. 7 shows a smaller neuron with a large number of nuclear masses of different sizes clumped together and looking as though the nucleus had fragmented. In these multinucleate neurons the nuclei are usually of different sizes and contain one or more nucleoli.

Mitosis has not been observed in these large neurons. They have many branched processes and move slowly. The processes are not readily drawn into the cell body, which is usually a precursor of mitosis in other kinds of cells, nor have the multinucleate cells themselves been seen to divide. It seems quite probable, therefore, that the nuclei of these multinucleate cells may have been formed by budding.

In the smaller brain cells, which were either glia or neurons (it was impossible to differentiate between them) mitosis was watched in the living cells. Fixed and stained preparations also showed different stages of mitosis. As the living cells began to divide, their long processes were drawn into the cell body. The nucleus elongated, chromosomes were seen in the metaphase, and were followed as they migrated to the poles of the spindle. The cytoplasm became very active and short blunt processes were formed at the periphery of the cell. The cell soon divided into two new cells. In one case the cell did not elongate at the time of division. A dividing plane passed across the cell and the two new cells remained *in situ* with very little change of shape.

DISCUSSION

The occurrence of nerve cells with two or more nuclei of equal or unequal size seems to indicate that nuclear division of some kind, without a corresponding cytoplasmic division, has been taking place. Whether this nuclear division was by mitosis or amitosis is not known. The fact that mitosis in the large neurons has never been seen and also that budding has been seen in the living cells and in the prepared slides is significant and suggests that the nuclei are increasing in number by amitosis. There is no evidence that fusion of two or more cells is taking place.

All the photomicrographs of living tissue cultures of human brain cells were made as routine records with a Leitz camera using 35 mm film.

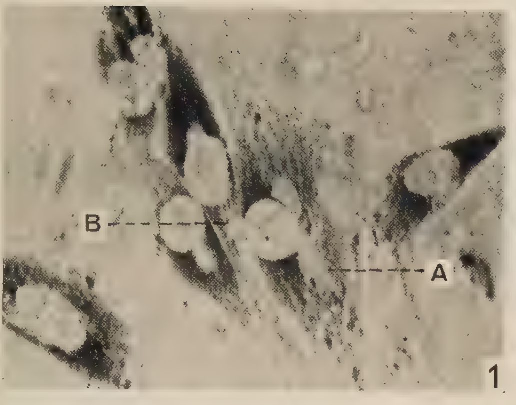
Fig. 1. Cells from a 34-day old culture of the pineal body of a fetus 142 mm C.R. Nuclear material is streaming into the cytoplasm at A in masses, at B through narrow channels. Nuclear buds are forming in two cells. × 335.

Figs. 2 and 3. Cells from a 31-day old culture of the medulla of a fetus 88 mm C.R. showing different stages of bud formation at C and D. × 170.

Fig. 4. Cells from a 31-day old culture of the medulla of a fetus 88 mm C.R. showing the pinching off of small globules from the nucleus at X. × 170.

Fig. 5. Twelve-day old culture from the cerebellum of a stillborn infant, showing a neuron with three nuclei, each containing one or more nucleoli. × 170.

Fig. 6. Eighty-two-day old culture from the cerebellum of a 135 mm C.R. fetus showing either a binucleate neuron or a neuron with a lobed nucleus. × 170.



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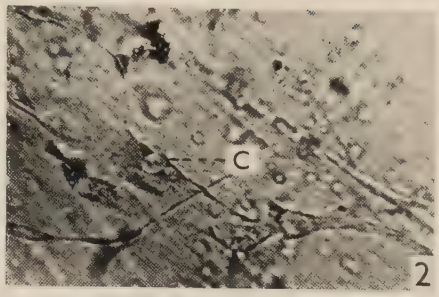

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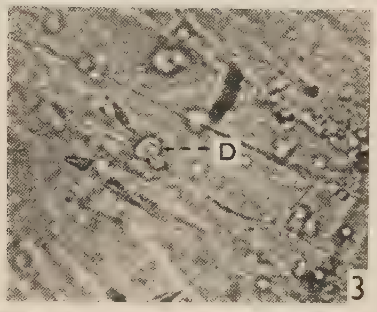

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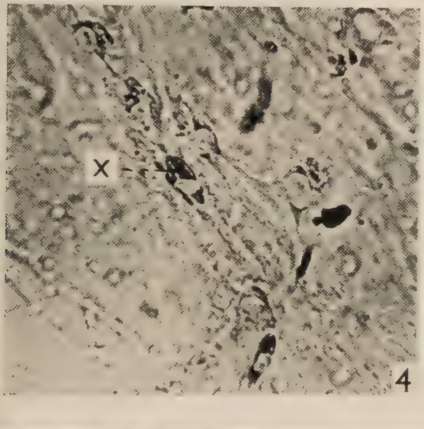

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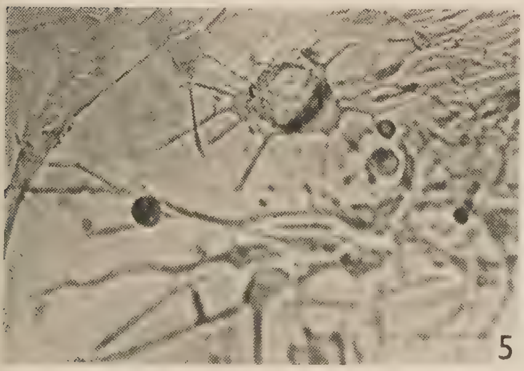
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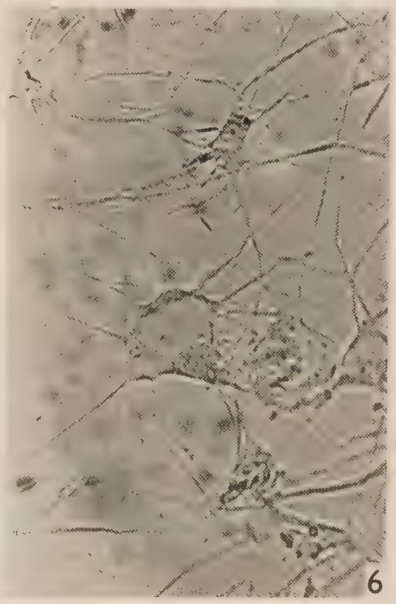
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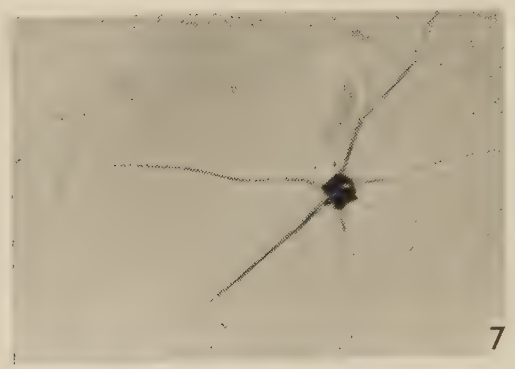
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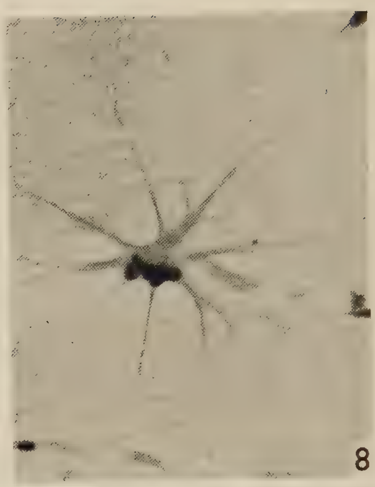
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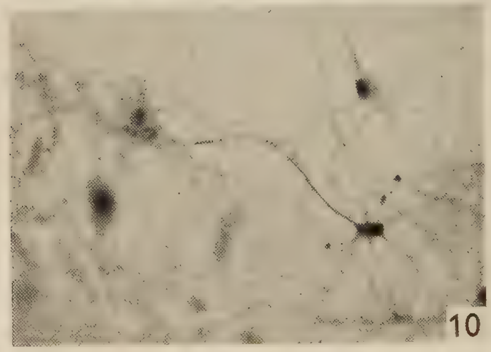
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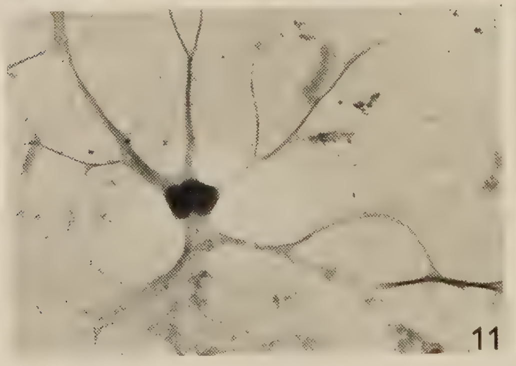
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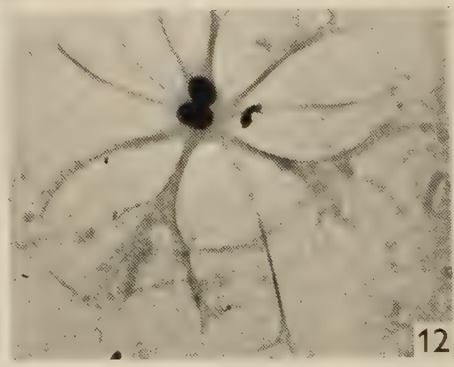
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In the multinucleate neurons seen in the present work, the nuclei are usually of different sizes which suggests amitosis by budding. The fact that there is not always an even number of nuclei present may also be considered an argument for amitosis when the nuclei are of nearly the same size.

The nuclei in a given cell do not seem to differ in density or in staining reaction, with one exception, fig. 8. Here a nucleus in the upper part of the cell appears much lighter than the other nuclei which are massed together in the lower part of the cell.

Furthermore, in these multinucleate cells the nuclei all seem to be in the same physiological condition, i.e., one of them is not normal in size and the others shrunken and pycnotic.

The distribution of the nuclei in these multinucleate cells is varied. Sometimes they are in a line (fig. 8). Again they are scattered irregularly about in the cell body (fig. 10); or they may be in a cluster or compact mass in the middle of the cell as in figure 7 where the number of nuclei is hard to determine.

The cause for the appearance of multinucleate cells in the tissues has been variously interpreted (1). Some investigators have found them more common in young tissues; others have found that they increase in number with age. Some consider them abnormal; others are sure they are not pathological. Here, in tissue cultures of human fetal brain cells, they are of such common occurrence in what appear to be healthy cultures that their presence is considered a normal phenomenon. Furthermore, since these multinucleate cells have all come from fetuses or new born infants, they cannot be considered a characteristic of old age. Even the cultures where they appeared are not necessarily old cultures.

Many investigators have reported finding multinucleate nerve cells in the sympathetic ganglia. Beaton, Holmes and Windle (1) found them in great

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Photomicrographs of nerve cells from human fetuses grown in tissue cultures. They were fixed, stained and then photographed with a Leitz camera using 35 mm film. All but figure 12 were made from the same slide which was a 53-day old culture of cerebellum from a 135 mm C.R. fetus. × 170.

Fig. 7. Many nuclear fragments or buds clumped together in the small body of a neuron.

Fig. 8. Seven or eight nuclei arranged in a row. One nucleus above the others takes a lighter stain.

Fig. 9. A nucleus with two buds of unequal size.

Fig. 10. A binucleate neuron, each nucleus with three nucleoli. Another small structure with a chromatin mass is present in the cell.

Fig. 11. Purkinje cell with two nuclei of unequal size. Each contains two nucleoli.

Fig. 12. Sixty-day old culture of the cerebellum of a 135 mm fetus. A Purkinje cell with two nuclei of unequal size.

numbers in the sympathetic ganglia of the human seminal vesicles, prostate gland and urinary bladder. Their paper contains an excellent bibliography.

Murray and Stout (5) studied adult human sympathetic ganglia cells in tissue cultures and found in old cells and in cells beginning to succumb that the nucleus might be lobate, it might fragment, or it might divide producing binucleate or even pentanucleate cells. They do not consider these cells as abnormal, since multinucleate sympathetic nerve cells are rather frequent in adult man (1). They too found no evidence of the fusion of cells to form multinucleate neurons nor did they ever see division of these multinucleate cells.

Williams (6) saw nuclei which might be "simple, lobulated, multiple, equal and unequal" in tissue cultures made from the hind brain of a 5-day old chick embryo.

However, as far as can be ascertained, the present work is the first to show multinucleate nerve cells from human brains.

SUMMARY

1. Nuclear material is poured out directly into the cytoplasm in masses and also through long narrow channels between the cytoplasmic granules.

2. Nuclear material is also given off in the form of small globules.

3. The nuclei form buds of different sizes, much larger than the globules, which are pinched off into the cytoplasm.

4. Cells are found with from 2 to 7 nuclei which are often of different sizes.

5. Nuclei in the same cell are usually of equal density and in the same physiological condition as shown by their staining reaction.

6. Mitosis was seen in small cells of the brain, probably glia cells.

7. Mitosis were not seen in large neurons which form many processes and move slowly. The processes are not readily drawn into the cell body, usually a precursor of mitosis in other kinds of cells.

I wish to express my thanks to Dr. Bachman and his staff at the University Hospital and to the Anatomical Board of the State of Pennsylvania for cooperation in obtaining material for this work. This work has been supported by a grant from the Special Research Fund of the University of Pennsylvania.

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AN ELECTRON MICROSCOPE STUDY OF THE LAMELLAR STRUCTURE OF CHLOROPLASTS

ERWIN STEINMANN[1]

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts

Received August 3, 1951

Polarized light studies of the large *Mougeotia* chloroplasts indicate that the birefringence is due chiefly to the form of the submicroscopic components, i.e., to the regular arrangement of layers which are thin with respect to the wave length of visible light. As the birefringence disappears or becomes extremely small when the refractive index of the imbibition fluid is 1.58 to 1.60, it seems probable that the layers consist mostly of proteins (6).

Investigations of the fine structure of retinal rods (10) and of the nerve myelin sheath (2, 11) have shown that such lamellar structures can be observed directly with the aid of the electron microscope, provided that a suitable fragmentation or thin sectioning technique is employed.

A similar lamellar structure has been suggested for the grana of the granular chloroplasts of the higher plants (3), in view of the discovery that only the single grana show birefringence (9). To a certain extent this could be proved by applying the electron microscope (5), because the unfixed grana easily overturn during the drying process on the supporting film and show the separated layers of which they are composed.

MATERIAL AND METHODS

We have used *Spirogyra* with a single spiral per cell and *Mougeotia*, both collected in ponds near Cambridge (Mass.) and kept for a few days in diffuse day-light to prevent an exaggerated starch formation.

We have found *Aspidistra elatior* to be a very suitable plant with which to confirm the lamellar structure of the grana. *Aspidistra*, a shade plant, has grana with a diameter greater than 0.5 μ.

Chloroplasts, like most lipid-protein systems, are very sensitive to changes in the environmental conditions. Electron microscope examination of the structure can be made only on fixed material. As could be expected from the birefringence experiments, osmium tetroxide in a concentration of 1 per cent gave the best results; it was the only fixative which preserved the lamellar structure of the chloroplasts.

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[1] This work was done during tenure of a fellowship of the American Swiss Foundation for Scientific Exchange sponsored by the International Institute of Education. Permanent address: Pflanzenphysiologisches Institut der ETH, Zürich, Switzerland.

The algal threads were fixed for a short interval, not exceeding 60 minutes, and then thoroughly washed with water. After having destroyed the cell walls with a blendor, a suspension of chloroplast fragments was obtained by using a sintered glass filter to separate cell walls from chloroplast fragments.

The suspension of fixed chloroplast fragments could be used for both thin sectioning and fragmentation with sonic vibrations: a) Thin sections were prepared after dehydration with alcohol and embedding in n-butyl methacrylate, cutting with a glass knife, as described by Geren and McCulloch (7). After removal of the plastic with toluene the preparations were shadowed with chromium. b) The suspension, after appropriate dilution, was treated with sonic vibrations of a frequency of 10 kc for 10 to 15 minutes. By centrifugation undestroyed material was separated from the fragmented chloroplasts which were then placed on a grid and shadowed with chromium.

The grana of *Aspidistra* were prepared according to the usual procedure (1, 8) with phosphate buffer, pH 6.4, or in some cases with distilled water.

An RCA electron microscope type EMU with intermediate lens was employed.

EXPERIMENTAL OBSERVATIONS

A. *Homogeneous algal chloroplasts:* As we are dealing with fixed chloroplasts we shall, to a certain extent, be unable to avoid the formation of artefacts. But the many sections we examined from OsO_4 fixed material invariably indicated a lamellar structure (Figs. 1, 2, 3). By removing the plastic from the section with toluene, the connection between the layers seems to become loose. In certain areas the chloroplast appears to be opened up (Figs. 2, 3). Relatively thick sections (0.3 μ) show this phenomenon much more frequently than very thin ones. In *Mougeotia* chloroplasts the splitting of the layered body is a common appearance (Fig. 3).

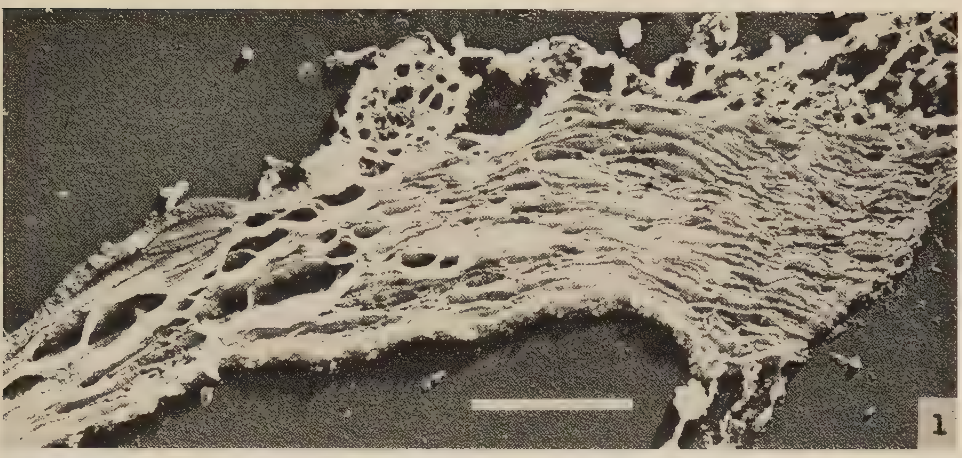
The structure of the pyrenoid, the locus of starch synthesis in the homogenous chloroplasts, is of special interest. The pyrenoid structure, well known from studies with the light microscope to consist of a protein nucleus surrounded by a sheath of subdivided starch bodies, could be confirmed, though the central protein part requires a more exact investigation of its detailed structure (Fig. 4). The starch does not reveal any regular fine structure in thin sections.

The mark on each figure corresponds to one micron.

Fig. 1. Cross section through *Spirogyra* chloroplast. Fixation: 10 minutes in 1 per cent OsO_4. 21 600 ×.

Fig. 2. Cross section through *Spirogyra* chloroplast. Fixation: 10 minutes in 1 per cent OsO_4. 23 400 ×.

Fig. 3. Cross section through *Mougeotia* chloroplast. Fixation: 45 minutes in 1 per cent OsO_4. 21 600 ×.



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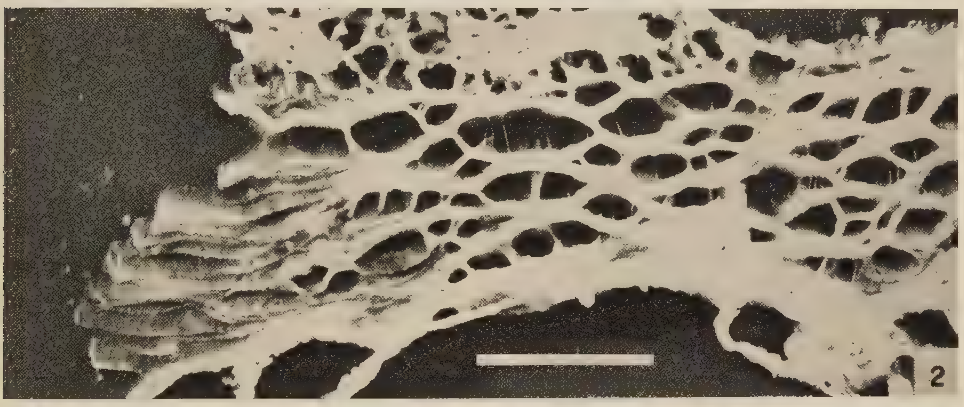

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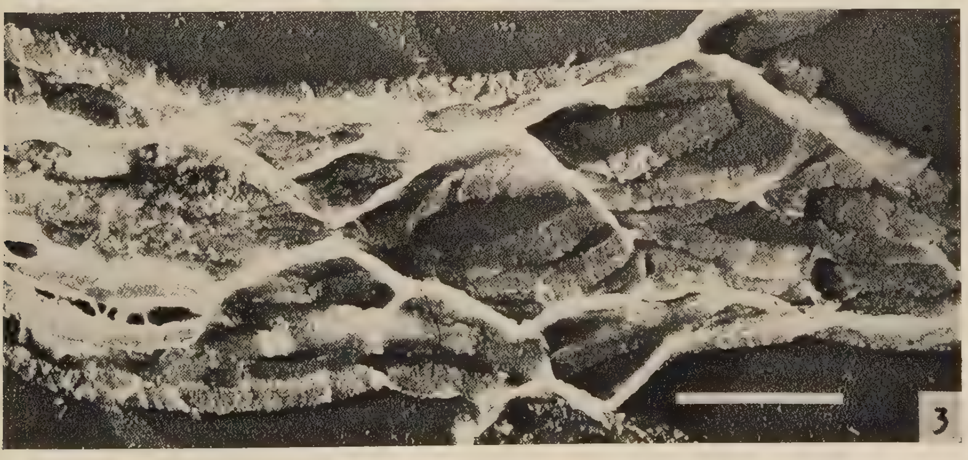

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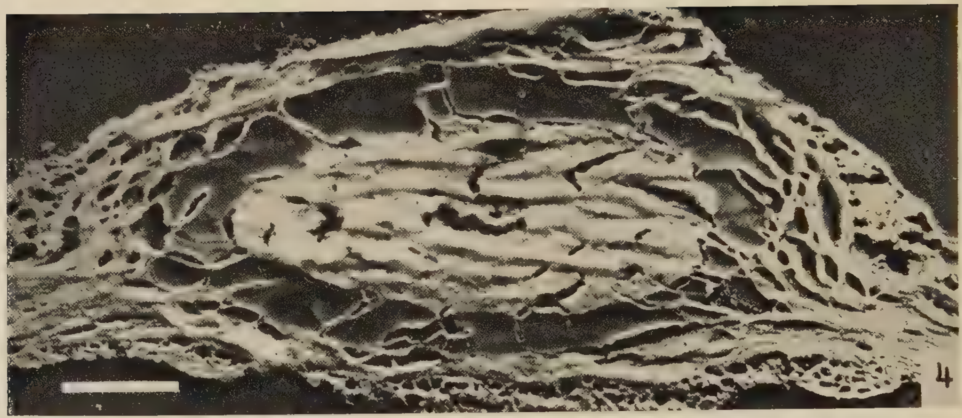

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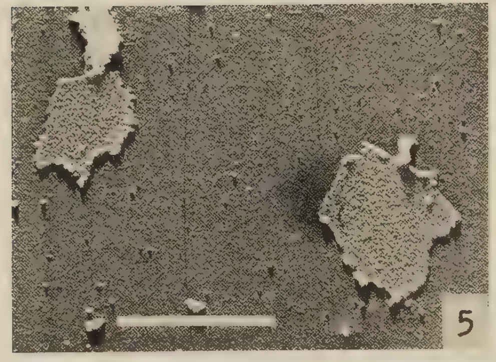

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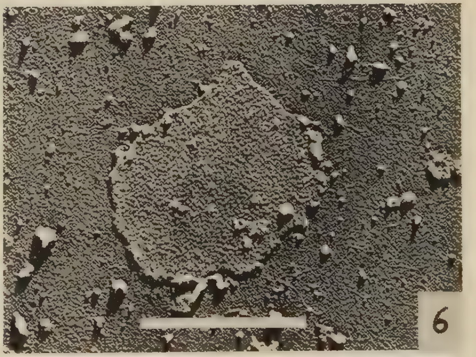

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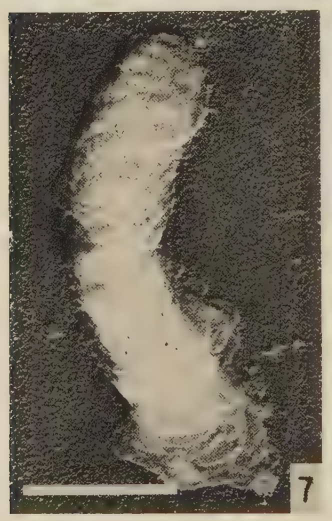

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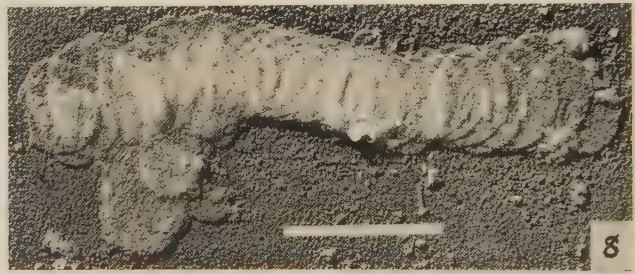

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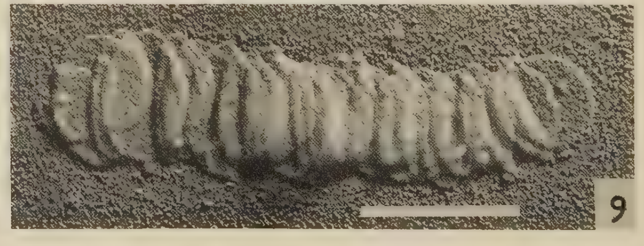

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Because as yet it has not been possible to measure accurately the thickness of a single layer in sections, we tried to obtain some more results fragmenting pure OsO_4 fixed chloroplast particles with sonic vibrations. Both in *Spirogyra* and *Mougeotia* preparations, the same irregularly shaped sheets could be observed which is consistent with the lamellar structure (Figs. 5, 6). Similar layers appear in preparations of retinal rods (10) and nerve myelin sheaths (2, 11) treated with sonic vibrations.

Measurement of the thickness of the thinnest of those lamellae, observed in 37 different *Spirogyra* preparations, has given as a result 69 ± 7 Å. As yet it is impossible to say anything about the exact chemical composition of these layers.

B. *Granular chloroplasts:* Figures 7, 8 and 9 show unfixed overturned *Aspidistra* grana either isolated with buffer (Fig. 7) or with distilled water (Figs. 8, 9). They suggest that the single granum is composed of a stack of layers forming a cylinder. The many layers of the *Aspidistra* granum apparently do not adhere very firmly to each other.

Preliminary experiments applying sonic fragmentation to OsO_4 fixed granular chloroplasts show thin circular layers with a diameter equal to the diameter of a granum and a thickness of about 70 Å, which seems to provide further evidence for the lamellar structure of the granum.

DISCUSSION

The results not only confirm the deductions from polarized light experiments that the chloroplasts contain very thin lamellae but show that these lamellae have a thickness of about 70 Å or multiple thereof. In the planes of the lamellae there is a fine punctate structure which may be due to a partial separation of one of the layer components (lipid) in these fixed preparations.

It may not be without significance that both the retinal rods and the chloroplasts, which have the common property of converting visible light into chemical energy, have the same type of lamellar ultrastructure. As yet, there

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Fig. 4. Cross section through a pyrenoid of a *Spirogyra* chloroplast. Fixation: 10 minutes in 1 per cent OsO_4. 15 300 ×.

Fig. 5. Layers obtained by sonic treatment of *Spirogyra* chloroplasts. Fixation: 15 minutes in 1 per cent OsO_4. 20 700 ×.

Fig. 6. Layers obtained by sonic treatment of *Mougeotia* chloroplasts. Fixation: 10 minutes in 1 per cent OsO_4. 21 600 ×.

Fig. 7. *Aspidistra* granum. Isolation with Sörensen phosphate buffer pH 6.4. 19 800 ×.

Fig. 8. *Aspidistra* granum. Isolation with distilled water. 20 700 ×.

Fig. 9. *Aspidistra* granum. Isolation with distilled water. 20 700 ×.

is little basis for speculation about the physical significance of this correlation. It is not impossible, however, that with the resolution now available with the electron microscope, it may be possible to observe morphological concomitants of certain phases of the photochemical process. The present observations form a basis upon which experiments along these lines may be devised.

SUMMARY

1. Thin sections and sonic fragmentation of OsO_4 fixed *Spirogyra* and *Mougeotia* chloroplasts examined with the electron microscope confirm the lamellar structure of these cell constituents.

2. The thickness of the lamellae, observed in preparations treated with sonic vibrations, was calculated from the shadow length to be about 70 Å.

3. *Aspidistra elatior* chloroplasts seem especially suitable for the demonstration of the lamellar structure of the grana.

We deeply appreciate the opportunity to carry out this work in the laboratories and under the direction of Prof. F. O. Schmitt. Likewise we wish to express our sincere thanks to all the members of the Biology Department for their constant help and kindness.

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THE MORPHOLOGY AND DEVELOPMENT OF MOLLUSCUM CONTAGIOSUM FROM ELECTRON MICROGRAPHS OF THIN SECTIONS[1]

W. G. BANFIELD[2], H. BUNTING, M. J. STRAUSS, and J. L. MELNICK

Laboratory of Pathology, Yale University School of Medicine, New Haven, Conn.

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The morphology of molluscum contagiosum has been extensively studied under the light microscope. The lesion usually described consists of a localized region of the epidermis in which the cells are altered by the presence of cytoplasmic inclusion bodies (the Henderson-Patterson molluscum body), that appear first in the Malpighian layer and reach maturity in the lower stratum corneum. Van Rooyen (11) has demonstrated by microdissection that the mature globular inclusion body is surrounded by a sheath and is filled with elementary bodies lying in a gelatinous matrix. Goodpasture (2) pointed out an enlargement of the nucleoli of early infected cells and also cytoplasmic particles surrounded by a halo; these particles he considered to be nucleolar material. He believed that in the initial infection the virus localized itself in the center of the cytoplasmic mass rather than being scattered throughout the cell. Rake and Blank (8) however, in tracing the development of the inclusion body, maintained that there is first a general increase in cytoplasmic ribonucleic acid which is followed by the appearance of diffusely scattered islands of desoxyribonucleic acid. The latter enlarged to form the characteristic inclusion body within which they described the ribonucleic acid compressed into septa. The elementary bodies from the supernatant portion of centrifuged material from the lesions of molluscum contagiosum have been found by electron microscopy to be brick-shaped (1, 8, 9, 10) with average measurements of 330 mμ by 230 mμ (10). Rake and Blank (8) examining the sediment from such preparations, demonstrated smaller particles measuring 100 mμ by 83 mμ which they suggest may be the precursors of the elementary bodies.

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[2] Fellow, American Cancer Society.

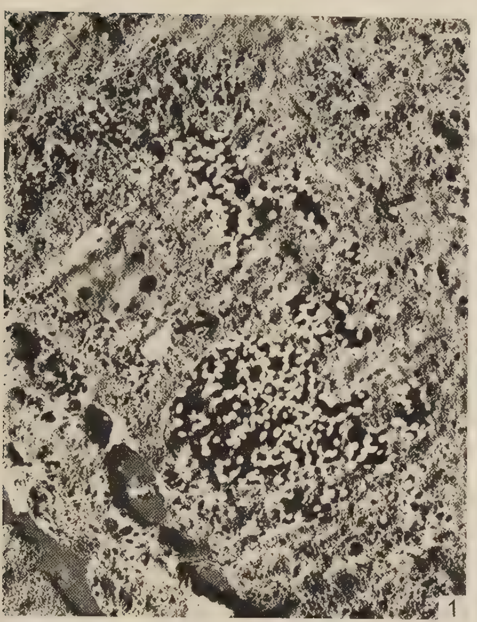
The present report extends the observations on the morphology and possible stages in the development of the virus by the examination of thin sections of molluscum lesions with the electron microscope.

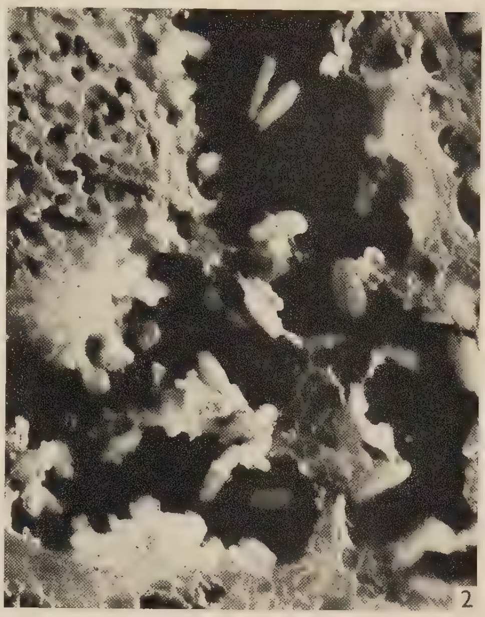
METHODS

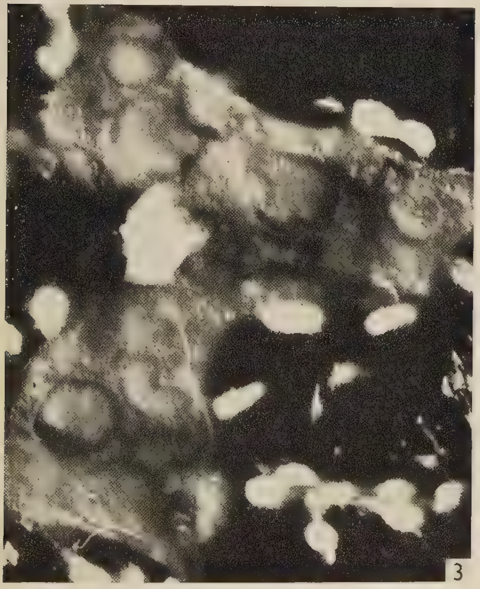
Four separate lesions of molluscum contagiosum freshly removed with a curette were fixed in 4 per cent buffered formalin for two to three hours. Pieces of tissue no larger than 1–2 mm on an edge were dehydrated in a graded series of alcohols and embedded in methacrylate (1 part methyl and 8 parts butyl), using 2–4 dichlorobenzoyl peroxide as a catalyst and incubated for 18–24 hours at 60° C (6). Sectioning was performed on a Spencer microtome adapted with a 1:20 wedge (7). The wedge and flywheel were counterbalanced and the microtome was driven by a motor (3). Sections were cut from 0.2 to 0.05 μ with a glass knife (5), mounted on collodion-covered grids, and, after removal of the methacrylate by xylol, were shadowed with palladium at an angle of approximately 1:7 before examination with an RCA electron microscope, model EMU. The illustrations are enlargements from negatives made by contact with the original plates.

RESULTS

In the prickle-cell layer of the epidermis, cells of the molluscum lesion were found that contained within their cytoplasm varying numbers of elementary bodies which did not appear to be confined within a membrane and were fewer in number than in the mature inclusion bodies. In these cells the virus particles seemed to have no particular arrangement or localization, but were distributed at random within the cytoplasm (Fig. 1). The shape of the elementary bodies, when only a few were present in the cell, often differed from that found in the mature inclusion bodies. Some were thinner in proportion to their length with typical measurements of 338 by 105 mμ, and at times were even lancet-shaped as shown in Fig. 2. The completely formed elementary bodies from the more mature inclusions were generally smooth and more ovoid in outline than the early forms, with average measurements of 360 by 210 mμ. These are similar in appearance and size to elementary bodies obtained from suspensions of ground molluscum lesions (Fig. 8). In one preparation round almost structureless cytoplasmic masses measuring up to 2.3 μ were frequently observed surrounded by particles of a size consistent with that of the elementary bodies. These masses may well represent the cytoplasmic "nucleolar" material described by Goodpasture (2). In several instances, elementary bodies were found associated with smaller particles. In one instance these particles closely resembled those pictured by Rake and Blank, but were somewhat smaller measuring 76 by 61 mμ (Fig. 12).







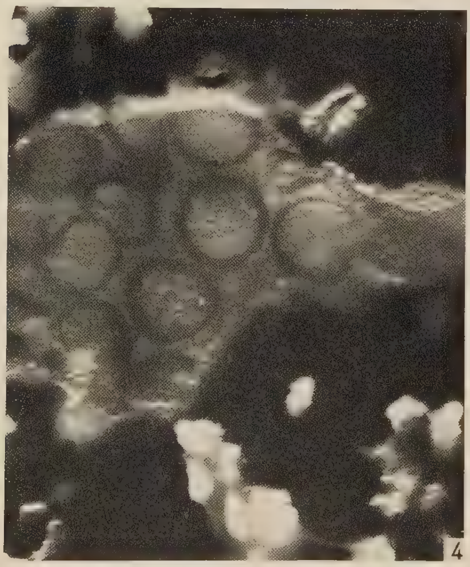
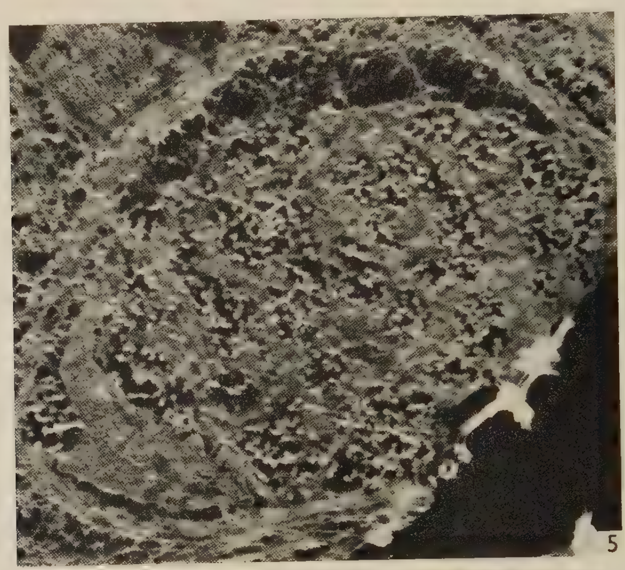


Fig. 1. An early infected cell with elementary bodies scattered diffusely throughout the cytoplasm. The nucleus and cell wall are indicated. 3 760 ×.

Fig. 2. Early infected cell. The lace-like structure of the nucleus is at the left with a portion of the cell wall at the right. Note the length of the elementary bodies in proportion to their width and the lancet shaped particles in the upper portion of the figure. 26 320 ×.

Fig. 3. A portion of a septum from a molluscum inclusion body showing segmentation of the matrix. Note the dark electron translucent zone surrounding the segmented areas and the marked indentation of several of these areas. The dense particles at the margins are more mature elementary bodies. 15 040 ×.

Fig. 4. Another example of segmentation in the septal matrix of an inclusion body. 26 320 ×.



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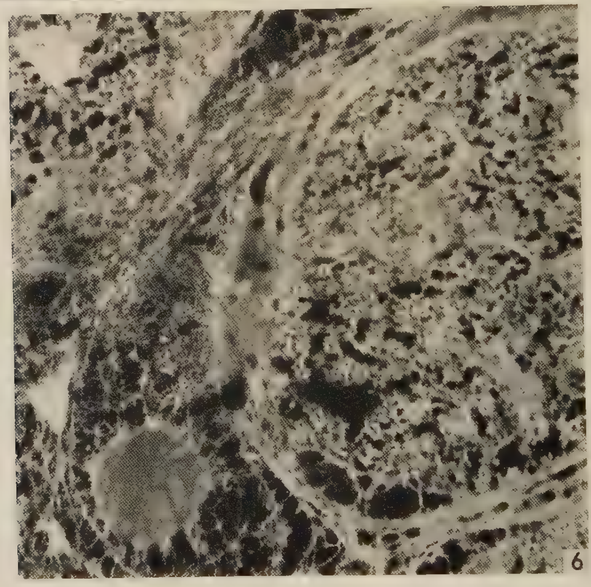

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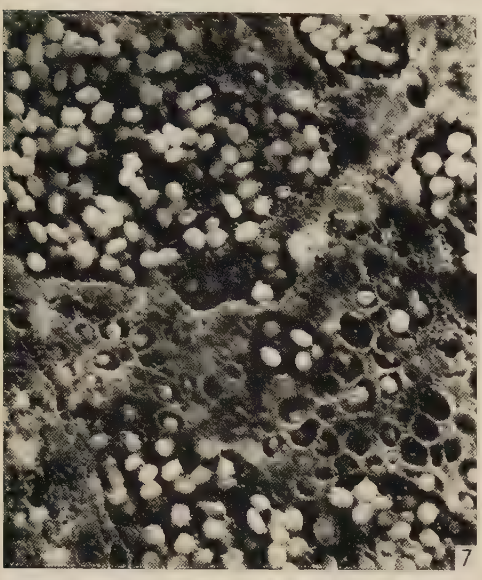
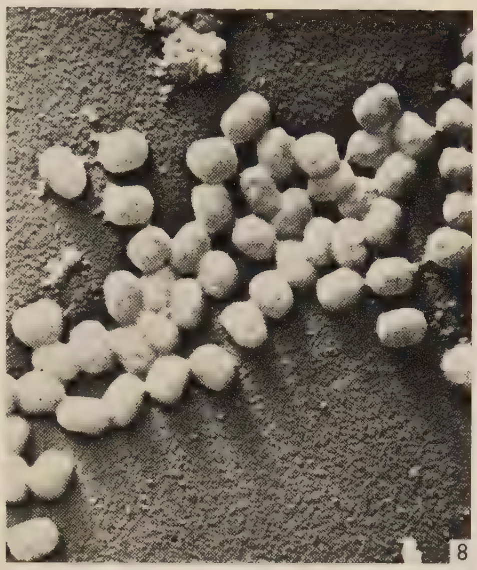
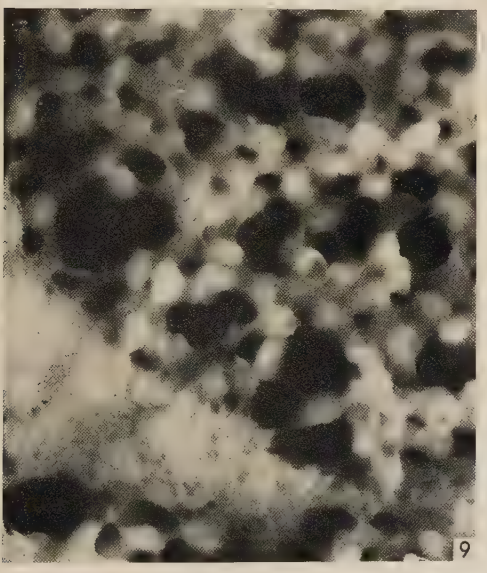
Fig. 5. Cell with mature inclusion body. The nucleus is pushed to one side (lower left) and the elementary bodies are in contact with the nucleus. 3 720 ×.

Fig. 6. Inclusion body with compressed nucleus. Note the clear demarcation between the inclusion and the nucleus. 3 255 ×.







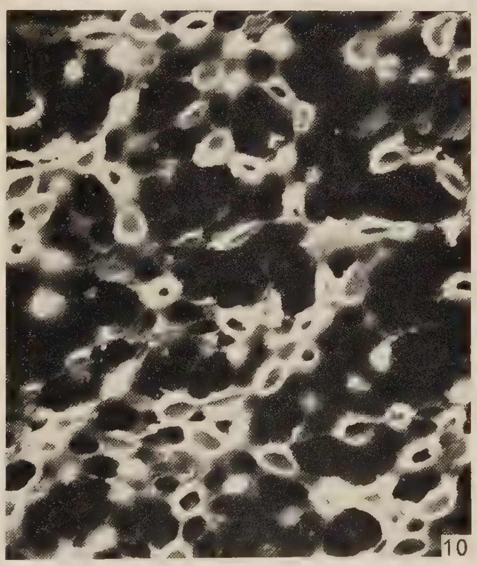
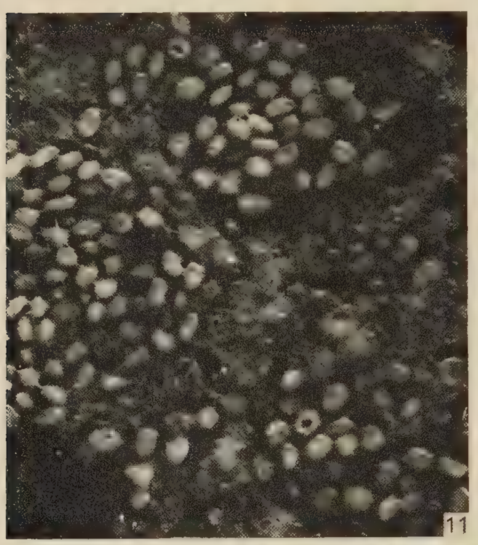


Fig. 7. A small portion of an inclusion body showing a septum with locules filled with mature elementary bodies on either side. Note the segmentation and variation of forms within the septum. 10 230 ×.

Fig. 8. Elementary bodies of molluscum contagiosum obtained from suspensions of molluscum lesions. 21 390 ×.

Fig. 9. Elementary bodies within a locule of an inclusion body. They are imbedded in an amorphous material which can be seen stretched between them. A portion of a septum is at the lower left of the figure. 21 390 ×.

Fig. 10. Sectioned elementary bodies shown at high magnification. Note the dense wall and apparently empty interior. 32 085 ×.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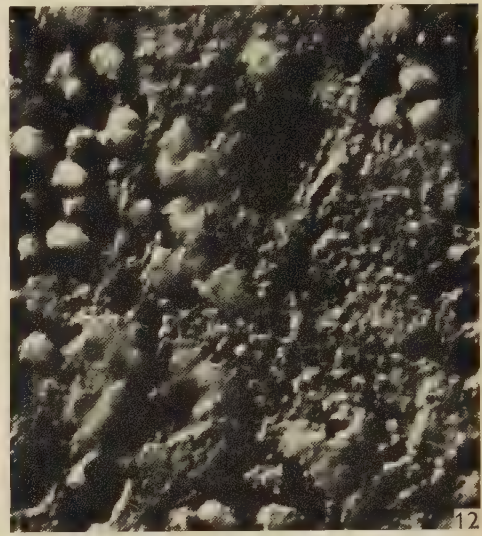
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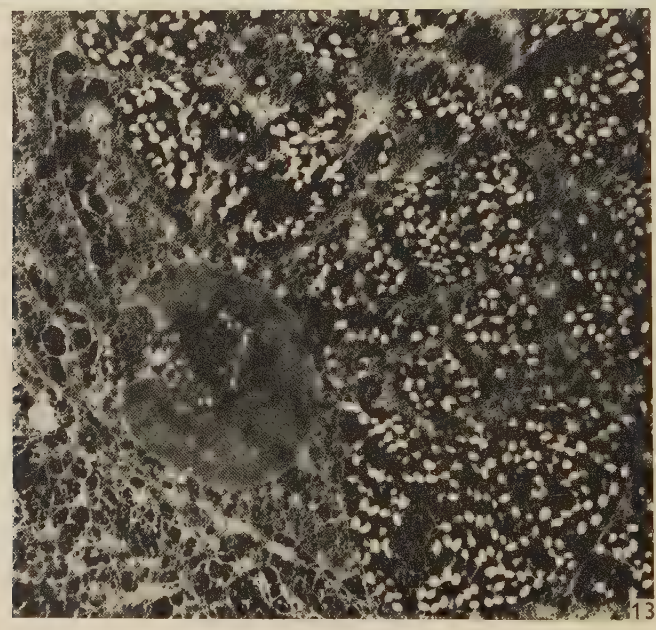
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Fig. 11. Elementary bodies sectioned and unsectioned. 12 150 ×.

Fig. 12. Elementary bodies associated with small particles that look almost identical with those of Rake and Blank though their measurements are somewhat smaller (76 by 61 mμ). 18 900 ×.

Fig. 13. Portion of cell with inclusion body and compressed nucleus. Note the size and structure of the nucleolus in contrast to the surrounding lacy nucleoplasm. 8 100 ×.

In Fig. 5 a mature inclusion body is shown occupying almost the entire cell with the nucleus displaced to one side. There is no distinct separation between the inclusion body and the nucleus that might indicate the sheath so conclusively demonstrated by Van Rooyen (10) with his microdissection technique. In those instances in which the separation between the nucleus and the inclusion body is definite (Fig. 6), it has not been possible to determine for certain whether the compressed nucleus is from the same cell or from another, lying either above or below the plane of section. In cells containing a large inclusion, the nucleoplasm has a lacy structure and is shrunken while the nucleolus holds its shape, is homogeneous, dense and, if anything, is increased in size (Fig. 13).

The largest inclusion bodies are divided into compartments by septa with mature elementary bodies filling the locules between the septa (Figs. 7, 13). In some instances areas of the matrix composing these septa appear to be cut out into rounded or indented structures (Figs. 3, 4, 7). Some of these bodies are distinctly contoured and separated from the surrounding matrix by a more electron-translucent zone as seen in shadowed preparations (Figs. 3, 4). All gradations in size and density can be found between these structures and the mature elementary bodies which would suggest that the latter may be formed by a segmentation and condensation of this matrix. In those early infected cells where only a very few elementary bodies are present no segmentation of matrix in the cytoplasm has been seen.

The elementary bodies in the locules of the larger inclusions are not always lying free, but often seem to be embedded in a material which surrounds certain elementary bodies and appears as stretched strands between others (Fig. 9). This may be the "gelatinous" material which Van Rooyen (11) demonstrated by microdissection. Sometimes the elementary bodies are arranged in strings, but in most instances there is no consistent pattern of orientation seen in the plane of the section.

Further observations have been made on the elementary body itself. In Fig. 10 there is a cluster of elementary bodies, most of which have been cut twice leaving only a central segment which appears as a dense ring of material with an empty center. The same ring-like forms are seen, even though the methacrylate has not been removed and shadowing has been omitted. That this is a real observation appears certain from the many sections in which both intact and sectioned elementary bodies are seen (Fig. 11). It is possible, then, that the elementary bodies of molluscum contagiosum, at least in these instances, possess a formed cortex and a less dense interior.

DISCUSSION

New concepts of the morphology and development of molluscum contagiosum have been suggested by this study of thin sections of molluscum lesions with the electron microscope. In addition this technique has facilitated further observations on some of the findings of other investigators.

Van Rooyen (11) has demonstrated a sheath about the older inclusions. In the electron micrographs, with the present method of fixation, the membranous structures that are seen are difficult to interpret as being identical with this sheath, inasmuch as they cannot positively be distinguished from possible artefacts of fixation and dehydration or from other membranous structures of the cell.

Elementary bodies have been searched for in nuclei but so far no more than suggestive instances of nuclear involvement have been seen.

The older inclusions were examined for a consistent pattern of orientation of the elementary bodies but none has thus far been observed. Though at times the elementary bodies occur in short strings this may well be an artefact of orientation produced during fixation, dehydration or by dessication of the "gelatinous" substance in which the mature elementary particles lie, or even by displacement during the process of sectioning.

Structural detail in the wall of the sectioned elementary bodies has not been seen. This wall appears to be of uniform density, of varied thickness and without demarcation between cortex and what might be adherent contents in those that are hollow. Though the central portion appears empty in some instances, in others, apparently sectioned, no hollow is seen. It is possible then that there is a difference in the central portion of elementary bodies which may depend upon their stage of development. This is further suggested by the fact that hollow central areas are seen consistently in some groups of elementary bodies but not in others. It must be kept in mind, however, that it may also be possible for the bodies either to be cut or pushed to one side depending upon the sharpness of the knife and their orientation to the cutting edge.

Epidermal cells with only a few elementary bodies in their cytoplasm may be considered to represent an early phase of infection. There are all gradations from these to other cells almost completely filled by a well-defined inclusion body. When an abundant matrix separates the elementary bodies, it is often segmented into areas which are in a graded series of sizes and densities to that of the mature elementary body. In the larger inclusions, this matrix remains as thin septa yet within them the same process of segmentation and condensation can be observed.

The septa are the thin hematoxylin-stained lines that can be seen crossing the inclusion body in hematoxylin-eosin preparations. Rake and Blank (8) have considered these septa to contain ribonucleic acid, a conclusion based upon staining with pyronin-methyl green. Kurnick (4), however, has shown that depolymerized desoxyribonucleic acid stains with pyronin similarly to ribonucleic acid. The locules between the septa, where the mature elementary bodies lie, contain desoxyribonucleic acid since these regions are stained by the Feulgen method (8). It seems apparent, then that the site of formation of the elementary bodies is in the material which is selectively stained with pyronin.

In several instances smaller particles have been found associated with the elementary bodies in the thin sections. It is not known what part, if any, they may play in the development of the virus. In one instance particles similar to but smaller (76 by 81 mμ) than those pictured by Rake and Blank (8) were seen (Fig. 12). The smaller size may be explained by the difference in methods of preparation. In the early infected cells many of the elementary bodies are thinner than most of those found in the mature inclusions and in addition lancet-shaped forms are seen. There is also an apparent absence of segmented matrix in the cytoplasm of these early infected cells which leaves the origin and fate of the early infection forms of the virus purely speculative. It should be noted that these latter bodies, which have been considered to be elementary bodies, differ in appearance from structures that have been interpreted as mitochondria in epidermal cells and further that they have never been seen in thin sections of control material from the epidermis examined with the electron microscope.

SUMMARY

Thin sections of molluscum contagiosum lesions from man have been examined with the electron microscope.

Cells were seen in the prickle cell layer of the epidermis which contain only very few cytoplasmic elementary bodies and are considered as early infected cells.

Cells were seen with varying numbers of elementary bodies scattered through the cytoplasm to cells entirely filled with elementary bodies in a mature inclusion.

The early infection forms of the virus differ somewhat from most of the elementary bodies seen in the larger inclusions.

The mature inclusion bodies are divided into locules by septa. The locules contain mature elementary bodies. The elementary bodies appear to form from the material composing the matrix of the septa by a process of segmentation and condensation.

The elementary bodies themselves have been sectioned and in some instances appear to have a formed cortex with a less dense interior.

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INTRACELLULAR LOCALIZATION OF ARGINASE IN HOMOGENATES OF RAT LIVER SUSPENDED IN DISTILLED WATER

A. H. SCHEIN and E. YOUNG

Department of Biochemistry, College of Medicine, University of Vermont, Burlington, Vermont

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We have recently reported on the intracellular localization of uricase in cell fractions prepared by differential centrifugation of rat liver homogenates suspended in distilled water or in hypertonic sucrose (6). It was found that 70–75 per cent of the homogenate uricase, as well as succinoxidase activity, was present in the mitochondrial fraction. The balance of uricase activity was found in the nuclear and microsomal fractions. In agreement with the work of others, pentose nucleic acid (PNA) was found in all of the cell fractions; desoxypentose nucleic acid (DNA) was present exclusively in the nuclear fraction. The morphology of the fractions, visualized by phase microscopy, was also described.

Employing the same techniques as previously, we have studied the intracellular localization of arginase in rat liver homogenates suspended in distilled water.

MATERIAL AND METHODS

The procedure for preparation of homogenates and differential centrifugation was essentially like that described by Hogeboom *et al.* (3). All operations were conducted in a cold room or refrigerated centrifuge (International, Model PR-1) at a temperature of from 1–5°. The arginase activity of the resulting nuclear, mitochondrial, microsomal and supernatant fluid fractions was measured by the photometric method of Van Slyke and Archibald (8). DNA and PNA concentrations were not measured.

RESULTS

The results of fractionations of livers from 5 adult male rats are incorporated in Table I. It may be seen that arginase is ubiquitously distributed among the cell fractions, with approximately 1/3 of the total homogenate activity in each of the nuclear and mitochondrial fractions and about 1/6 in

TABLE I

Arginase activity of cell fractions.
The results represent amount per 100 mg of fresh tissue or its equivalent.

Tissue Fractions	Nitrogen %	Arginase Activity			
		urea formed mg	% of homogenate	Specific Activity	
				ug urea/ug N	Ratio
Homogenate..........	100[1,2]	102±5[2]	100[1,2]	29±1.5[2]	1
Nuclear Fraction......	14± .7	36±3.4	36±3.7	73±5.2	2.5
Mitochondria	27±1.7	31±2.4	31±2.5	33±1.6	1.1
Microsomes...........	15±1.6	16±2.8	15±2.7	31±3.2	1.1
Supernatant Fluid	38±1.3	12±1.2	12±1.2	9± .8	.3
Recovery			93±3.0		

each of the microsomal and supernatant fluid fractions. The specific activities, however, calculated on a nitrogen basis reveal important differences in distribution. Thus, the nuclear fractions has 2 $\frac{1}{2}$ times the arginase activity of the homogenate; the mitochondrial and microsomal fractions have approximately the same activity as that of the homogenate. The supernatant fluid fraction is relatively low in arginase activity.

One possible reason for the appearance of appreciable amounts of enzyme in all of the fractions could be the solubilization of arginase from its primary site or sites followed by occlusion or adsorption on particulate matter. Dounce and Beyer, however, showed that adsorption of added beef liver arginase by cytoplasmic granules of homogenized lamb kidney was negligible (2), for the supernatant solution remaining when the cytoplasmic constituents had been spun down at 18 000 RPM contained practically all of the added arginase. Although there was some adsorption of added arginase by isolated nuclei, it was of insufficient magnitude, they felt, to appreciably affect the quantity of arginase as measured in the nuclei.

We have carried out an experiment designed to test the effect of repeated washings on the arginase content and concentration of the mitochondrial fraction.

Forty-five ml of an aqueous 10 per cent homogenate suspension from which the nuclear fraction had been removed, was divided among 7 tubes and centrifuged at 13 700 × g for 11 minutes. The supernatant fluids containing microsomes and

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[1] Homogenate taken as 100 per cent. Average values calculated from individual determinations.
[2] Figures following ± are standard errors of the mean.

TABLE II

Effect of repeated washings on arginase activity of mitochondria.

	Nitrogen[1] ug	Urea produced[1] mg	Specific Activity[2] ug urea/ug N
Unwashed mitochondria	875	22	25
Washed 1 × »	860	22	25
» 3 × »	805	21	26
» 5 × »	740	18	24
» 6 × »	700	17	24

soluble materials were discarded. One of the precipitates representing unwashed mitochondria was set aside in the cold. Three ml of water were added to each of the others and the mitochondria resuspended and resedimented as above. Again one of the precipitates, representing once-washed mitochondria was set aside and the other 5 precipitates washed as above. This was done for a total of 6 times. The unwashed and washed mitochondria were made up to 10 per cent suspensions and were separately tested for arginase activity (Table II).

It can be seen that there is almost no loss of specific activity with repeated washing, thus it is unlikely that arginase was either occluded or weakly adsorbed by mitochondria. Material so retained would probably be washed away to a measurable degree. It is of interest to observe that only 20 per cent of the mitochondrial fraction nitrogen was removed by 6 washings. It is not possible to state whether some of the mitochondria had been lysed or whether an increasing failure to sediment a portion of the mitochondria developed. Possibly some of the mitochondrial nitrogen was becoming soluble. If the latter were true, soluble nitrogen and arginase nitrogen were lost in the same proportion since the specific arginase activity did not change appreciably on washing.

DISCUSSION

Shortly after our experiments were completed Ludewig and Chanutin reported on the distribution of arginase in fractions of rat liver homogenates suspended in isotonic sucrose (4). Their general technique of centrifuging and their method of measuring arginase activity were also patterned after

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[1] per ml of 10 per cent mitochondrial suspension. Barring washing losses 1 ml contained the equivalent amount of mitochondria present in 100 mg of fresh tissue.

[2] Liver derived from an adult female rat. We have twice observed that arginase specific activity is lower in females than in males. The results, however, are not conclusive in this small series of experiments.

Hogeboom *et al.* and Van Slyke *et al.* respectively. These investigators, employing rats of the Wistar strain, measured arginase activity of fractions of livers from animals which had been subjected to whole body x-irradiation and their non-irradiated controls. X-irradiation had no effect on the activity or distribution of arginase. Their results, when compared to ours, show marked similarities of arginase distribution in the nuclear and supernatant fluid fractions. There was a difference, however, in the mitochondrial and microsomal fractions (14.8 and 27 per cent of the homogenate activity respectively), whereas our figures are 31 per cent for the mitochondrial and 15 per cent for the microsomal fractions.

Some factors which may account for these variations in cellular arginase distribution are:

1. The differences in per cent recovery: Our recoveries of 93 per cent of homogenate arginase activity in the fractions are 10 per cent higher.
2. The differences in suspending media, isotonic sucrose vs. distilled water.

In this connection mention should be made of the difficulty referred to by Muntwyler, Seifter and Harkness (5) and also Schneider and Hogeboom (7) of separating the (tan) mitochondrial pellet from overlaying loosely packed microsomes (pink-white) in sucrose. We have experienced this difficulty in aqueous suspensions and the second sedimentation of the mitochondria does *not* sharply separate mitochondria and microsomes in this medium, as it apparently does in sucrose. Thus, contamination of the mitochondrial fractions by microsomes is favored. While our average nitrogen values for mitochondrial and microsomal fractions closely approximate those of Ludewig and others, wide individual variations did occur.

3. *Strains of rats:* Wistar rats were used by the Virginia investigators. Our rats were of the Sprague-Dawley strain. The difference in results, if valid, might possibly help to explain known metabolic differences existing among strains of rats and this facet of enzyme distribution should be further investigated.

Dounce (1) has stated that the enzyme arginase appears to be a *real* constituent of nuclei of rat liver cells and that nuclei contain arginase in a concentration at least as high as in cytoplasm. Our results with the nuclear fraction (which also contains unbroken cells and cytoplasmic constituents) offer additional evidence for this theory. In addition, the high activity of the nuclear fraction on a nitrogen basis indicates that nuclei are *especially* rich in arginase.

CONCLUSION

In summary, we (as well as Ludewig) have reported the presence of arginase in all of the liver cell fractions. The total concentrations and specific activity of arginase in the nuclear fraction was too high to be caused merely by large or small granule contamination and therefore nuclei represent a major site of arginase activity. That arginase is also associated to some extent with particulate matter of the cell is clear, since both the total concentration and specific activities of arginase of the mitochondrial and microsomal fractions are appreciably higher than the supernatant fluid fractions.

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NOTE ON MECHANICAL MEANS OF DISRUPTING CELLS[1]

A. R. T. DENUES

The Sloan-Kettering Institute for Cancer Research, New York, N. Y.

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In connection with studies of the identity of isolated chromosomes, radically different means of disrupting cells were sought for use in preparations of the type made by Claude and Potter (1) and by Mirsky, Pollister, and Ris (2, 3, 4). This note will summarize results of an examination of a variety of means for this purpose. The performance of the Logeman Hand Colloid Mill will be emphasized.

These studies were made with clean chicken erythrocytes obtained from oxalated blood by centrifugation. The cells were washed in physiological saline or in Gey's solution. Systematic counts were made of the numbers of intact cells C, of free nuclei N, and of free threads T seen by phase contrast microscopy in wet mounts after various treatments. Cells and free nuclei were readily counted, of course. Free threads were conservatively scored: counts were made only of threadlike bodies a few micra in length, dotlike bodies being neglected. These threads were of quite uniform twisted appearance. On application of aceto-orcein they were found to stain well. The results were expressed as two convenient process parameters, used in this note for brevity, *viz.*, N/C and T/N. (These statistics are not to be thought of as having biological meaning!)

Among miscellaneous means examined, grinding of thawing cells with sand (*cf.* Claude) was tried. In four runs, the weight-ratio of sand to cells ranged from 1 to 4 and the time of grinding from 3 to 15 minutes. Varying results ensued. Insufficient reduction was shown in one run by a good yield of nuclei; apparently excessive grinding was shown in others by uniform yields of dotlike objects, less than 1 micron in diameter; masses of good threads taking aceto-orcein stain were obtained in one run. In view of these erratic results, the method was not extensively pursued.

A few experiments were made with a Potter-Elvehjem homogenizer (5). Up to 30′ treatment of a 1:1 suspension of cells in Gey's solution at 1 000 rpm failed to rupture many cells. Some nuclei and a few threads resulted, but attempts to separate any mass of free chromatin threads failed. Similar treatment using a ground glass homogenizer with conical seat likewise failed to rupture many cells. Such methods were therefore thought unpromising in this application.

An improvised rubber and glass hand homogenizer, reported satisfactory for liver and kidney (6), was also found unpromising for the disruption of these red cells in a

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[1] This work, done at the Detroit Institute of Cancer Research, was supported in part by research grants from the National Cancer Institute, of the National Institutes of Health, Public Health Service, and from the American Cancer Society, Inc.

1:1 slurry. Up to 10′ of operation with tight and loose homogenizers gave but a few free nuclei and threads (N/C = .3, T/N = .7 at best).

Two high-speed impeller-type mixers were tested, *viz.*, the "Waring Blendor" (*cf.* Mirsky *et al.*) and the "Whiz Mixer." The latter is essentially similar to the Blendor but was found to include two undesirable fiber washers in its hub assembly. It was not, therefore, extensively tested. A problem of contamination developed with these devices: fine particulate matter appeared and the 2 600 A-absorption of the fluids increased with treatment. This contamination appeared to come chiefly from the hub assembly. It could be rather satisfactorily eliminated in the Blendor by cleaning the dismantled assembly and replacing the one fiber washer with a brass washer. Initial tests of the Blendor were made with a 15:1 dilution of cells. No intact cells remained after six-1′ runs separated by cooling periods, but unruptured nuclei persisted even after 30′ of such operation; the maximum ratio T/N was 29. Tests of the effect of dilution on these results indicated that increasing this from 15 × to 70 × and, in another series, from 5 × to 15 × to 100 × increased the ratios T/N progressively and by several fold. A practical high dilution, as 20 ×, has thus been employed in subsequent work.

A survey of colloid mills was made from a chemical engineering viewpoint. Among the larger mills, the jacketed centrifugal smooth-surface type used by Mirsky and Ris (3) appeared uniquely appropriate. However, such high-speed mills may include a torsional action. They are also so costly as to be of restricted availability. Interest was therefore concentrated upon the smaller Logeman Hand Colloid Mill. This apparently promised relatively mild treatment by shear with little turbulence or torsion. The Logeman Mill is of stainless steel construction and consists essentially of a spring-loaded valve through which fluids can be forced by a piston hand pump. Relatively simple shear is thus effected in the valve seat. Of models available, the 1/2-liter Mill with single valve proved most useful. A larger (2.9-liter) model with two valves and pumps was also extensively tested, but with less success. Promising results were obtained in initial tests of the 1/2-liter Mill as received: 15-fold extrusion of a thick slurry of cells and 30-fold extrusion of slightly diluted suspensions of cells showed progressive disruptions of cells and nuclei to yield many threads mixed with few nuclei and virtually no intact cells.

The disruption of cells and of nuclei was regularly improved by increasing the dilution to 25 × (figure 1). In subsequent runs, further dilution through 100 × and 440 × to 1 000 × showed the same favorable trends to continue (table I). A practical result with cells eliminated and with T/N in the order of 100 was attained with a convenient dilution of 100 × in about 10 extrusions.

The effect of increasing the load on the valve was developed in a further series of runs. Using 100 × dilution of cells and spacers to load the spring

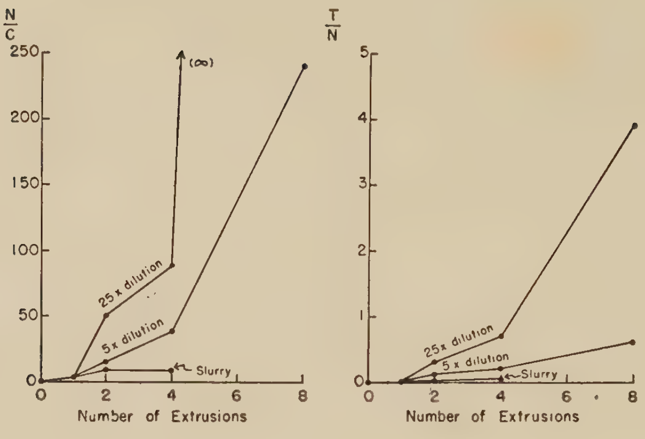

Fig. 1. Initial effects of dilution on the disruption by Logeman Mill of chicken red cells *C* to yield free nuclei *N* and free chromatin threads *T*.

TABLE I

Effects of Dilution on the Disruption of Chicken Red Cells by the Standard $^1/_2$-liter Logeman Mill.

Dilution of Cells	Run	N/C[1] after extrusion no.				T/N[1] after extrusion no.			
		1	4	8	12	1	4	8	12
Slurry	I	5	65	190	—	0	.1	.1	—
	II	5	—	—	—	0	—	—	—
	III	15	180	—	—	0	.1	.2	—
25×	I	70	∞	∞	∞	.2	—	3	5
	III	20	∞	∞	∞	.1	.2	5	∞
100×	I	20	∞	∞	∞	.9	1.5	5	00
	II	8	∞	∞	∞	.1	9	18	—
	III	15	∞	∞	∞	.2	25	75	150
250×	II	∞	∞	∞	∞	.1	9	14	—
	III	75	∞	∞	∞	.2	35	∞	600
440×, 500×	II	40	∞	∞	—	0	24	32	—
	III	210	∞	∞	∞	.3	20	50	∞
1 000×	III	270	∞	∞	∞	.4	∞	∞	∞

[1] C = number of intact cells.
N = number of free nuclei.
T = number of free chromatin threads.

TABLE II

Effects of Increasing Load on the Extrusion Valve on the Disruption of Chicken Red Cells by the Logeman Mill.

Number of $^1/_8$″ Spacers	N/C after extrusion no.			T/N after extrusion no.		
	1	4	8	1	4	8
0	10	∞	∞	.1	2	7
1	20	∞	∞	.2	17	200
1	25	∞	∞	.1	5	25 *a*)
1	40	∞	∞	.2	8	190
2 *b*)	17	∞	∞	.1	2	15

a) = Tangles of threads present, not counted.
b) = Spring-loading cap retracted to obtain flow.

TABLE III

Disruption of Chicken Red Cells by the Logeman Mill under Selected "Standard" Conditions *a*).

Number of Extrusions	N/C *b*)		T/N *b*)	
	Run A	Run B	Run A	Run B
1	70	4	.6	.4
2	—	∞	—	2
4	∞	∞	100	17
6	∞	∞	200	130
8	∞	∞	450	680
10	*c*)	∞	∞	∞

a) Cells diluted 100 ×; one $^1/_8$″ spacer on 1″–18 AWG spring.
b) C = number of intact cells.
N = number of free nuclei.
T = number of free chromatin threads.
c) N = C = 0.

supplied with the equipment, it was found that one 1/8th inch spacer led to real improvements. However, further compression of the spring by an additional spacer required retraction of the spring-loading cap to obtain reasonable flow, thus precluding further improvements with the available pump. T/N in the order of 200 was attained here (table II). Use of springs wound from heavier steel wires gave no great improvement in another series of runs, as it again became necessary to retract the spring-loading cap. The reality of this practical limitation was emphasized by results of the same order of magnitude obtained with five different springs set empirically to obtain practical flows.

Confirmatory runs under the "standard" conditions thus selected, with cells at 100 × dilution and with a 1″-No. 18 AWG spring with a 1/8″ spacer, gave excellent results (table III). The preparations showed homogeneous fields of the characteristic threads that again stained well with aceto-orcein. In another run, using nuclei prepared by freezing and thawing the cells, 1, 4, 6, 8, and 10 extrusions at 100 × dilution gave successive ratios T/N of 1, 6, 10, 37, and 130. The usefulness of the Logeman Mill for the purpose at hand is apparent from these data.

The assistance of Mrs. G. G. Leithauser in this work is gratefully acknowledged.

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AN INVESTIGATION OF PROTEINS DURING THE DEVELOPMENT OF THE AMPHIBIAN EMBRYO

REED A. FLICKINGER[1] and GEORGE W. NACE[2]

Laboratoire de Morphologie Animale, Université Libre de Bruxelles, Bruxelles, Belgique

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Recently much attention has been focused on the role of proteins in embryonic development. This question has been investigated by means of *in vitro* serological and electrophoretic techniques and by the action of antisera upon developing embryos. The present study attempts to gain a further understanding of the temporal-spatial relations and possible functions of the proteins by employing these techniques.

MATERIALS AND METHODS

First and second year oocytes remaining in the ovary after ovulation (small oocytes), mature ovarian oocytes (large oocytes), blastulae (stage 9), neurulae (stages 14–15) and hatched tailbuds (stages 20–21) of the frog, *Rana temporaria*, were used in these investigations. During the early part of the season the embryos were obtained by pituitary injections. Both spawned out ovaries and those containing mature oocytes were perfused with saline and the mature oocytes, in their follicular membranes, were dissected from the ovaries. Blastulae and neurulae were obtained by removing fertilized eggs from the jelly and allowing development to proceed to the desired stages, while tailbuds were collected immediately after hatching. These materials were washed with buffer and then either frozen and stored at –15° C, lyophilized (9) or used immediately.

The fractionation procedures used at these stages varied somewhat between the serological and electrophoretic experiments; however, the basic prodecure is described in Figure 1. The supernatant, vitellin, pseudoglobulin (livetin) and pigment fractions were obtained from large oocytes and hatched tailbud stages; only the supernatant fractions were obtained from the other stages. Aside from these fractions pooled male and female adult frog sera and testes homogenate were also prepared.

Certain of these lyophilized materials were made up as 2 per cent protein[3] solutions in phosphate buffer, pH 7.3, and utilized as injection antigens. Each antigen was injected into two young rabbits, each of which received one series of inoculations

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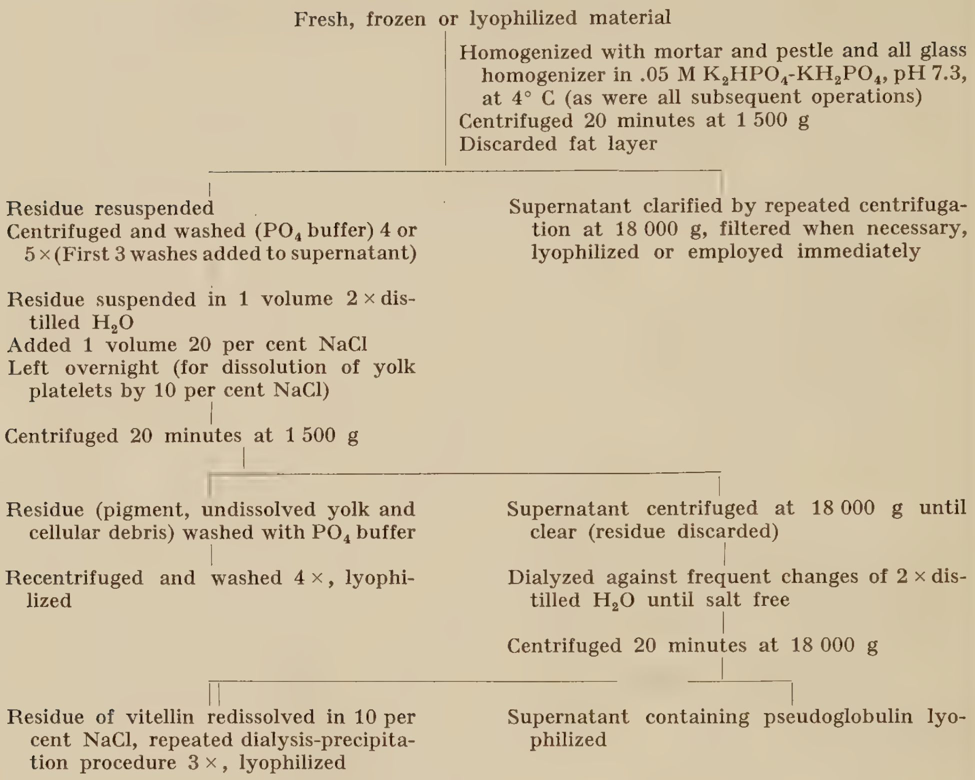
[1] Rockefeller Research Fellow, 1950–1952. On leave from: Zoological Laboratory, University of Pennsylvania, Philadelphia 4, Pennsylvania.

[2] Public Health Service Research Fellow of the National Institutes of Health, 1950–1951. Present address: Zoology Department, Duke University, Durham, North Carolina.

[3] Total nitrogen determinations were made according to the nesslerization procedure described in Umbreit *et al.* (18, p. 103) and protein concentrations were calculated with the factor 6.25.

Fig. 1. *Fractionation Scheme.*



Fresh, frozen or lyophilized material

Homogenized with mortar and pestle and all glass homogenizer in .05 M K_2HPO_4-KH_2PO_4, pH 7.3, at 4° C (as were all subsequent operations)
Centrifuged 20 minutes at 1 500 g
Discarded fat layer

Residue resuspended
Centrifuged and washed (PO_4 buffer) 4 or 5 × (First 3 washes added to supernatant)

Supernatant clarified by repeated centrifugation at 18 000 g, filtered when necessary, lyophilized or employed immediately

Residue suspended in 1 volume 2 × distilled H_2O
Added 1 volume 20 per cent NaCl
Left overnight (for dissolution of yolk platelets by 10 per cent NaCl)

Centrifuged 20 minutes at 1 500 g

Residue (pigment, undissolved yolk and cellular debris) washed with PO_4 buffer

Recentrifuged and washed 4 ×, lyophilized

Supernatant centrifuged at 18 000 g until clear (residue discarded)

Dialyzed against frequent changes of 2 × distilled H_2O until salt free

Centrifuged 20 minutes at 18 000 g

Residue of vitellin redissolved in 10 per cent NaCl, repeated dialysis-precipitation procedure 3 ×, lyophilized

Supernatant containing pseudoglobulin lyophilized

following the schedule of Nace and Schechtman (10). None of these rabbits possessed sera containing natural antibodies to these antigens as determined by interface precipitin reactions. A total of 5.25 ml of vitellin, pigment, and testes suspensions were injected intraperitoneally, while 3.35 ml of the supernatant fractions and adult frog sera were injected intravenously. Either nine or ten days after the last injection the rabbits were starved for 12 hours and bled from the ear. After preliminary tests for antibody activity, the appropriate antisera were pooled, Seitz filtered, frozen and stored at —15° C.

OBSERVATIONS

The effect of the various antisera upon embryonic development: The exposure of developing embryos to various antisera is not only a very sensitive means of studying the changing protein pattern during development, but is also of interest since it allows one to experimentally correlate these changes at the biochemical level with morphogenesis itself. For example, is it to be expected that antibodies bind and inhibit the specific action of antigens which might normally partake in such embryonic phenomena as determination and differentiation? To this end these antisera were put into the media with developing embryos, injected into one blastomere at the 2 cell stage and into the blastocoel at stage 9, and neural crest cells were explanted in their presence. The blastomere and blastocoel injections were performed, not only because of the difficulty of removing the vitelline membranes from the earlier stages, but also in order to introduce the antisera into their intra and intercellular environments.

Preliminary experiments in which embryos were allowed to develop in various dilutions of normal rabbit serum indicated that more concentrated dilutions could be tolerated only after dialysis of the serum against Holtfreter's solution. It was found that the antisera which possessed the antigen end point titers shown in Table I retained homologous activities after a 1/25 dilution of the dialyzed antisera. In the hope of attaining the most effective antisera concentrations with a minimum of biological injury, antisera dilutions of 1/6.25 were employed in these experiments. These antisera were diluted with .3 per cent Holtfreter's for embryos and .9 per cent for blastomere and blastocoel injections and neural crest cultures. In several cases dilutions of 1/12.5, and solutions of $(NH_4)_2SO_4$ precipitated and dialyzed immune globulins, were also utilized. The crystallized potassium salt of penicillin G (4 oxford units/ml), dihydrostreptomycin (sulfate) (40 γ/ml) and Elkosine (.01 ml/ml) were added to the antisera to prevent infection as merthiolate (1/10 000) was found to be toxic to the embryos. Elkosine contains sodium 6-paraminobenzenesulfamide-2,4-dimethylpyrimidin in an amount which corresponds to 200 mg/ml of the free acid. It was necessary to neutralize this solution.

All antisera shown in Table I (except anti-testes) were injected[1] into one of the first two blastomeres and resulted in localized cleavage retardation at the morula stage. This was followed by cytolysis and the subsequent appearance of a large injury site on the injected side of the embryo at the tailbud stage. Since normal rabbit serum acted similarly it must be assumed that the injury effects noted were due to non-specific action of the sera. This response was not due to injection injury alone as controls injected with Holtfreter's solution developed normally. Injection of these antisera into the blastocoel at stage 9

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[1] We are indebted to Dr. S. Gothié who performed these injections. She employed the Micromanipulateur de Fonbrune to inject approximately 5×10^{-3} mm^3 into each blastomere and 5×10^{-2} to 5×10^{-1}mm^3 into each blastocoel, after first removing an equivalent amount of blastocoel fluid. In all, ten series of experiments, utilizing 15 to 20 embryos with each antiserum, were carried out.

Table I.

Results of experiments with *Rana temporaria* precipitin systems.

Antigen end point titers are expressed as reciprocals of antigen dilutions; undiluted antigen was .5 per cent protein.

Test antigens[1, 2, 3]

Antisera to	Absorbed with	Small oocyte super-natant (SOS)	Large oocyte super-natant (LOS)	Large oocyte vitellin (LOV)[4]	Large oocyte pseudo-globulin (LOP)	Blastula super-natant (BS)	Neurula super-natant (NS)	Tailbud super-natant (TBS)	Tailbud vitellin (TBV)[4]	Tailbud pseudo-globulin (TBP)	Adult frog testes	Adult frog serum (AFS)
AFS	Unabsorbed....	**500**[5]	100	100	3	10	10	10	100	3	10	1 000
LOS	Unabsorbed....	**1 000**	500	0[6]	10	100	100	100	10	100	**3**	100
	AFS	500	100	0	10	10	10	100	10	0[6]	**3**	0
	AFS, SOS	0	±[7]	0	0	**3**	0	1	0	0	—	0
LOV	Unabsorbed....	**500**	100	500	100	10	10	**500**	100	10	—	10
	AFS	**500**	10	100	10	**10**	**10**	100	**100**	10	0[6]	0
Large oocyte pigment (LOPig)	Unabsorbed....	**500**	**500**	100	10	100	**500**	**500**	100	100	—	—
	AFS	100	10	**100**	10	10	10	100	**100**	10	0[6]	0
	AFS, TBPig ...	**3**	**3**	0	0	0[8]	0[8]	1	0	0	—	0
TBS	Unabsorbed....	**500**	100	**1**	100	100	100	500	10	10	0[6]	100
	AFS	**500**	10	**1**	10	10	10	100	**10**	1	—	0
	AFS, SOS	0	3	0	0	0	0	**1**	0	1	—	0
	AFS, SOS, LOS	0	0	—	0	0	0	**3**	0	1	—	0
	AFS, SOS, LOS, LOPig	—	—	—	—	—	—	**3**	—	1	—	—
Tailbud pigment (TBPig)	Unabsorbed....	**500**	100	100	100	100	100	100	100	10	—	—
	AFS	100	10	**100**	10	±	10	**100**	**100**	**10**	0	0
	AFS, SOS	0	±	10	0	0	0	0	10	0	—	0
	AFS, SOS, LOS	0	0	**10**	0	0	0	0	**10**	0	—	0
	AFS, SOS, LOS, LOPig	—	—	0	—	—	—	—	0	—	—	—
Testes	Unabsorbed....	—	**100**	—	—	—	—	10	—	—	**1 000**	**100**
	AFS	100	10	0[6]	0[6]	**100**	10	10	0	0	10	0

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[1] Normal rabbit serum and saline controls were uniformly negative. [2] Antigen dilutions utilized: 1, 3, 10, 100, 500 and 1 000.

[3] "—" signifies no test was made; "0" signifies negative results.

[4] Antisera tested against LOV and TBV were adjusted to 10 per cent saline with crystalline NaCl.

did not impede morphogenesis; the few abnormalities observed were due to defective gastrulation resulting from injection injury.

In order to test the effect of these antisera on cell movement and differentiation *in vitro*, a number of neural crest cultures were carried out (16) with each antiserum. Only a small number of good cases were obtained, probably due to an unsatisfactory explantation medium. However, normal amoeboid dispersal, yolk platelet break-down and pigmentation of cells were observed in antisera to large oocyte supernatant, large oocyte vitellin, tailbud pigment and adult frog serum.

Demembranated gastrulae (stages 11–12) and neurulae (stages 14–17) were placed in the antisera[1] (active complement added in several experiments), and embryos in normal rabbit serum and in Holtfreter's solution, acted as controls.

Both the experimental and control embryos developed at a normal rate and normal feeding tadpoles resulted with one notable exception. The antiserum to hatched tailbud supernatant (stages 20–21), diluted 1/6.25 with .3 per cent Holtfreter's solution, blocked development at stage 19–20 in six series of experiments. At this stage the embryos showed cytolysis of the epidermis along anterior-posterior, dorsal-ventral gradients and usually died a day or two after the initial appearance of cytolysis. In all cases, including one in which stage 19–20 embryos were added to the anti-tailbud supernatant, cytolysis occurred at stage 19–20. Thus, it seems that length of exposure is not a crucial factor. In one experiment no effect of this antiserum upon axolotl, *Amblystoma mexicanum*, embryos was observed. When both stages 15–16 and 19–20 embryos were present in the same culture dish for the same length of time, only the tailbuds underwent cytolysis. If the cytolysis was due to infection one would expect cytolysis of the neurulae as well. Since these fractions were probably not pure, one might expect some action of anti-tailbud pigment upon the embryos. However, as indicated in the section dealing with *in vitro* serological reactions, this antiserum has no activity to tailbud supernatant and therefore had no effect upon the embryos. Controls and animals from the anti-TBS[2] experiments were stained with methylgreen pyronine, but did not reveal any striking change in their cellular basophily. It appears probable that the antibody does not gain access to the interior of the cells, but reacts solely with the antigens of the cell membranes.

One may conclude from these experiments that a new antigen(s) or combining group(s) appears between the large oocyte and tailbud stages. That it appears at the surface of the embryo at stage 19–20 was manifested by the cytolysis at that stage; that it is not a large oocyte antigen, which moves to the surface, is apparent since none of the large oocyte antisera gave this effect. Although it can be detected at this stage, this antigen may arise earlier, but not be available to the antibody. It is impossible to determine from these experiments if this cytolysis indicates the existence of an antigen gradient or merely a susceptibility gradient to a toxic agent.

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[1] Anti-testes was not used in the biological experiments.

[2] Text abbreviations are defined in Table I.

Brachet (3) has found that antisera to large, medium, and small granules and supernatant of the tadpole produced effects similar to those described here. He also noted ciliary and muscular immobility prior to the appearance of cytolysis and obtained reversal of the inhibition if the embryos were placed in Holtfreter's solution before the advent of extreme cytolysis. Anti-large granule serum, which had the highest titer, caused the most pronounced biological effects in these experiments.

Interface precipitin reactions: In order to further elucidate the time sequence of antigen appearance the test antigens and antisera, shown in Table I were used in interface precipitin reactions (*cf.* 10) in conjunction with serial absorptions. The various fractions, obtained according to Figure 1, were suspended in and dialyzed against .9 per cent saline, containing 1/10 000 merthiolate, except for the vitellin fractions in which merthiolated 10 per cent saline was used. The starting material was the lyophilized powder except for SOS, BS and NS which had been stored in the frozen state and were utilized immediately after fractionation. These were centrifuged at 18 000 g and diluted to .5 per cent protein concentration, which was the standard (1/1) test antigen dilution.

Merthiolated antisera were used unabsorbed and after absorption. Absorption was accomplished by the addition of repeated small quantities of the lyophilized powders or solutions of certain antigens. These mixtures were agitated at room temperature for 2–3 hours, refrigerated at 4° C for 2–3 hours or overnight, centrifuged and tested for completion of absorption. This procedure was repeated until the test indicated the complete removal of activity to the absorption antigen after which one more aliquot was added. In no case did the dilution exceed one part of absorption antigen solution to three parts of antiserum; for the most part dilution was much less than this.

An examination of Table I shows that the unabsorbed antisera reacted with all antigens tested, except in two cases where the most concentrated dilution was not tested (reciprocal reactions were positive in these two cases). The reduction of titers by AFS absorption indicated the presence of serum or serum-like combining groups in all preparations. The small and large oocyte preparations, although obtained from perfused ovaries, undoubtedly contained some serum, as did the hatched tailbuds which possess circulating blood. However, BS and NS, which were also effected by this absorption, could not contain serum contamination and therefore must have serum-like combining groups as indicated by Cooper (7). The retention of activity to all stages after AFS absorption demonstrates the presence of other common reactive groups throughout embryonic development. The high activity of the SOS antigen(s) to the antisera, both before and after AFS absorption, should be noted. Although the data are not sufficient for interpretation, this may somehow be related to the active synthetic processes of this period. AFS absorbed anti-testes reacted with the supernatant fractions, but not with the yolk, indicating that an antigen(s) common to the testes is in the supernatant fraction. Thus, a non-serum-like antigen(s) of the embryo is also common to the adult.

The activity of the various fractions of a given stage illustrated their lack of purity from a serological point of view. However, the low titers of the vitellin preparations to anti-supernatants of the corresponding stages, as compared with the reciprocal reactions, indicated that supernatant preparations contained very little vitellin contamination. However, vitellin fractions were more highly contaminated with supernatant antigens. In spite of the presence of these supernatant contaminates in the yolk preparations, there were some indications of differences in the specificity of the water soluble pseudoglobulin and the supernatant antigens.

SOS absorption of AFS absorbed antisera to LOS, TBS and TBPig demonstrated the presence of an antigen(s) in the supernatant fractions which is not present in SOS. Tailbud pseudoglobulin also reacted with the SOS and AFS absorbed anti-TBS. This was the only positive reaction of this type and since SOS absorption removed the activity to all other pseudoglobulin fractions, it seems probable that the test antigen, tailbud pseudoglobulin, was contaminated with supernatant. However, the appearance of a new antigen(s) common to the mature oocyte and tailbud stages is clearly demonstrated by the reaction of the supernatant fractions with the SOS and AFS absorbed antisera.

Continuing the serial absorptions of the anti-TBS and TBPig, these antisera were absorbed with LOS. From this absorption, evidence presented itself that TBS contains an antigen(s) distinct from that found in LOS. The positive reaction of tailbud pseudoglobulin with absorbed anti-TBS can be explained as in the case of SOS absorption. The absence of activity of these SOS and LOS absorbed antisera to BS and NS can be understood if one assumes that the new antigen arises after neurulation. However, it is difficult to explain the apparent absence of BS and NS activity with the SOS absorbed antisera, since it is already known that an antigen is present in the mature oocytes which is not present in the small ones. One may hypothesize concerning possible modifications of the antigens at the blastula and neurula stages. These might include an alteration of protein configuration, solubility changes or perhaps a modification of the relative concentration of the antigens present. Large oocyte pigment absorption (tested for completion of absorption with LOV) of the serially absorbed anti-TBS did not alter the anti-TBS reactions. It is apparent that the new TBS component is found only in the tailbud supernatant.

Both LOV and TBV activity were removed from anti-TBPig by LOPig absorption. It is to be emphasized that the pigment fraction contained much undissolved yolk and therefore this absorption is equivalent to a vitellin absorption, which was not performed because of the high salt content of this preparation. Conversely, when AFS absorbed anti-LOPig was further absorbed with TBPig, all activity to the vitellin and pseudoglobulin fractions was removed. It is seen that the antigens in these fractions are serologically stable from the mature oocyte to the tailbud stage. The LOPig absorption demonstrated that no new antigen(s) arises between these two stages and that the large oocyte vitellin fraction is serologically identical to that of the tailbuds. In this regard it is interesting to note that spectrophotometric measurements upon lyophilized vitellin, extracted with 5 per cent perchloric acid (room temperature), demonstrated the marked absorption peak at 2 600 Å characteristic of the nucleic acids. Equal amounts of large oocyte and tailbud vitellin were extracted and three times as much specific absorption was observed with the tailbud extracts.

Oudin reactions: Precipitin reactions closely following the Oudin method (11) were carried out with the unabsorbed and AFS absorbed antisera. The antisera were suspended in columns (1.5 × 45 mm) of dilute agar into which the test antigens were allowed to diffuse. By this method precipitate bands are formed which represent the minimum number of antigens in the system. The results were in agreement with the interface precipitin reactions and were of equal sensitivity. One to three bands were seen in all tests with the exception of the AFS homologous system in which six bands were observed. Both long and short diffuse bands, sometimes without sharp anterior boundaries, were observed in a number of cases. Aside from the AFS system, the number of bands in these experiments was consistently less than found by Cooper (7). These diverse results may perhaps be due to differences in antisera strength or to methods of antigen preparation. Further experiments are necessary for adequate interpretation of these data.

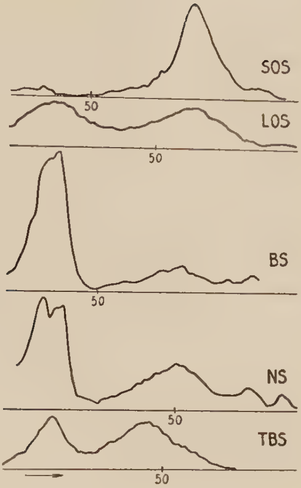

Fig. 2. Electrophoretic patterns of embryo supernatants. Anodic migration, 1.8–2.0 milliamperes, 38 volts, 25 minutes duration. Ordinates expressed as Δ 5 (1), Abscissae in mm migrated.

Electrophoresis: In the hope of obtaining electrophoretic patterns, which might be correlated with the serological phase of this investigation, SOS, LOS, BS, NS and TBS were analyzed with the micro-electrophoresis apparatus of Antweiler (1). The preparation of these materials varied slightly from the procedure outlined in Fig. 1. The fresh or frozen material was homogenized at 4° C in a minimal amount of electrophoretic medium[1] (pH 7.1, μ .105) against which the first supernatant was then dialyzed. The lyophilized material was taken up directly in the medium and then dialyzed. The protein concentrations of these dialyzed solutions were adjusted to 1 per cent by dilution with electrophoretic medium. In a few cases it was necessary to evaporate the solution by suspending the material in a dialysis bag over a fan in the cold room (4° C). These solutions were then redialyzed and adjusted to 1 per cent protein with the exception of BS which was .5 per cent.

A series of thirty-six electrophoretic experiments was made under various conditions, including a few experiments with lyophilized material, a pH of 8.6, and cathodal migration. Patterns obtained with the lyophilized material did not differ significantly from those shown in Figure 2; the only effect of higher pH was to alter relative mobilities of the components and in no instance was cathodal migration of materials observed. The curves presented in Fig. 2 were obtained with supernatants prepared from frozen, unlyophilized material. To facilitate interpretation the patterns have been arbitrarily aligned on the abscissa since there was considerable variation in mobility with preparations from the same stage. This may be due to unknown differences in the fractionation procedures or perhaps may be attributed to the apparatus.

An examination of the curves illustrates the presence of two major components

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[1]

KCl	.055 Molar
NaCl	.025 M
K_2HPO_4	.007 M
$NaH_2PO_4 . H_2O$	.003 M

in the mature oocyte, blastula, neurula and tailbud preparations, but only one in the small oocyte. Thus the electrophoretic evidence points to a new component in the mature oocyte which is not present in the small oocyte. That these components are similar throughout the stages of development studied is attested to by experiments in which equal amounts of the supernatants from different stages were mixed in various combinations. In all cases two major peaks were observed in the patterns resulting from these mixture experiments. This leads to the conclusion that these peaks correspond from one stage to another; in the case of the small oocyte only one of these constituents is present.

To determine if the microsomes contributed to these components, experiments were carried out with ultracentrifuged (60 000 g for 1 hour) mature oocyte supernatant. After ultracentrifugation a reddish layer of high density, whose color persisted for only a day or two, was noted just above the pellet at the bottom of the tube. However, the usual two LOS peaks were demonstrated with this material and also with the suspended pellet, i.e., microsomes. There was some indication from the size of the peaks that the slower electrophoretic component was concentrated in this red, high density fraction.

DISCUSSION

Both the biological and *in vitro* serological experiments demonstrate the appearance of a new antigen(s) by the tailbud stage. The electrophoretic data correlate with the *in vitro* serological results by showing a new component in the mature, as compared with the first and second year oocytes. The failure of electrophoresis to demonstrate the new tailbud component may be related to the different manner by which these techniques characterize large molecules. The new tailbud material may be quantitatively impossible to detect electrophoretically or may possess the same mobility as constituents already present. This component might be revealed by experiments utilizing other pH values.

It is of some interest that these new components arising in early development are detected in the supernatant fractions. Therefore, they are either water soluble or reside in particles not sedimented at 18 000 g, i.e., microsomes. Brachet (2) stresses the role of the microsomes in developmental and synthetic processes and his unpublished work, quoted previously in this paper, suggests that antisera to the granules are most effective in inhibiting development. This suggests the possibility that the new LOS and TBS antigens may reside in the microsomes and further, that they may be synthesized there.

A significant amount of absorption in the ultraviolet at 2 600 Å was observed in the vitellin fractions. That this absorption is not primarily due to ribonucleoproteins may be inferred from the work of Brachet and Chantrenne

(4), who demonstrated a greater quantity of free ribonucleoprotein in the immature oocyte than in the later stages where much of it is bound to microsomes. The original centrifugation at 1 500 g, which separated the pigment and yolk from the supernatant, would have left practically all the microsomes in the supernatant fraction. Thus vitellin fractions, which were repeatedly washed, were probably microsome-free. That this absorbing substance may be largely nucleohistone (DNA-protein) is evidenced by its greater abundance in the tailbud as compared with the mature oocyte and this would be a logical result of chromosome reduplication during growth (*cf.* 5). The vitellin fractions remained serologically stable throughout the period of development studied and one must therefore assume that nucleohistone is not an active antigen or that it maintains its specificity during this period.

In attempting to determine the morphological distribution of the new TBS antigen(s), the biological experiments would lead to the conclusion that it resides in the epidermis, but one must bear in mind that the surface reactions may only reflect the availability of the antibody to the antigen. It is unlikely that the epidermis is permeable to such large molecules. The possible means of antisera entrance into the embryo by stage 19–20 would include being carried into the invaginating archenteron or being trapped in the neurocoel; however, negative cytological results seem to deny any significant antigen-antibody reactions within the embryo. This may mean the new antigen is absent at these sites or that an insufficient number of active antibody molecules are available to the new antigen to produce a biological manifestation.

One might expect a sequential appearance of antigens in the embryo according to the concept of changing specificity during development. In this regard, our work, and that of many others, tends to confirm this assumption. Perlmann and Gustafson (13) have demonstrated a new antigen(s) at gastrulation in the sea urchin, *Paracentrotus lividus*. Burke *et al.* (6) have presented evidence revealing changes in the antigenic patterns of amphibian and chick embryos, while evidence from work on the chick embryo in Schechtman's laboratory (14) also confirms this thesis. As indicated previously our work supports that of Cooper (7) in finding serum-like combining groups in the amphibian embryo, although the new LOS and TBS antigens described here were demonstrated after absorption with adult frog serum, and hence would not be comparable to the serum-like constituents. Ten Cate and Van Doorenmaalen (15) detected both amphibian and chick lens antigens shortly before morphological differentiation of the lens, but they imply that refined techniques probably would demonstrate these antigens in still younger stages.

The specific morphological significance of the new LOS and TBS antigens is difficult to deduce from these data. That the new TBS constituent does play a morphological role is illustrated by the cytolysis and death of the animals in the *in vivo* serological experiments. Parks (12) has described a similar response on the part of tadpoles exposed to antisera directed against saline extracts of feeding tadpoles. The biological action of his antisera was dependent upon the presence of complement, while the anti-TBS, which had been Seitz filtered and stored in the frozen state, certainly did not contain complement. Thus it is likely that these investigations have not dealt with comparable antigen systems. This view is further supported by the age difference between the animals used in these two studies. A similar type of experiment, dealing with the effects of antisera upon development, has been carried out on chick material by Ebert (8). Certain organ antigens were demonstrated as early as the primitive streak stage and some specific morphogenetic effects were noted, e.g., inhibition of heart formation by anti-adult heart serum.

It is to be hoped that our growing knowledge of antigens of the embryo will soon permit further experiments and correlations dealing with their precise role in morphogenesis, perhaps along the lines suggested by Tyler (17) and Weiss (19).

SUMMARY

1. Serological and electrophoretic methods have been utilized to investigate the temporal and spatial relations of proteins during the development of *Rana temporaria*.

2. Antisera to mature ovarian oocyte supernatant, vitellin and pigment fractions, the supernatant and pigment fractions of hatched tailbuds and to adult frog sera were utilized in the biological experiments. They were injected into one of the first two blastomeres and the blastocoel, introduced into neural crest cultures and into media in which embryos were developing. The only specific effects observed were with anti-tailbud supernatant. Gastrulae or neurulae cultured in dialyzed and diluted anti-tailbud supernatant showed an inhibition of development, gradient-wise cytolysis and death at stage 19–20. These results demonstrate the presence of an antigen(s) which arises between the mature ovarian oocyte and tailbud stages and which resides in the epidermis at stage 19–20.

3. Unabsorbed and appropriately absorbed antisera were used in interface precipitin reactions with test antigens from immature ovarian oocytes, mature ovarian oocytes, blastulae, neurulae and tailbuds. Both serum-like

and non-serum-like combining groups were found throughout development; the vitellin fractions were serologically identical in the mature ovarian oocyte and tailbud stages; and two new antigens were detected, one in the mature ovarian oocyte supernatant, and the other in the tailbud supernatant.

4. Electrophoresis of the supernatant fractions provided evidence for the existence of two major components throughout development, with the exception of the immature ovarian oocytes where one major component was observed.

5. The essential agreement of the biological, *in vitro* serological and electrophoretic experiments indicates the appearance of at least two new proteins during the course of development studied and these have been discussed in relation to some of the previous literature.

We wish to thank Professor Jean Brachet for his interest in our work and for extending to us the generous hospitality of his laboratory.

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THE ROLE OF THE CORTICAL GRANULES IN THE FORMATION OF THE FERTILIZATION MEMBRANE IN EGGS FROM JAPANESE SEA URCHINS. I

YOSHIYUKI ENDO

Biology Department, Tokyo Metropolitan University, Meguro-ku, Tokyo, Japan

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Under the surface of the mature echinoid egg, there is a single layer of characteristic small granules—the cortical granules—which disappear at fertilization. According to Motomura (6), the fertilization membrane of echinoids consists of the vitelline membrane of unfertilized egg plus the substance of the cortical granules. Runnström (7–10) in particular has during the later years studied the behavior of the cortical material, including the cortical granules, under various experimental conditions, in order to clarify the mechanism of the formation of the fertilization membrane.

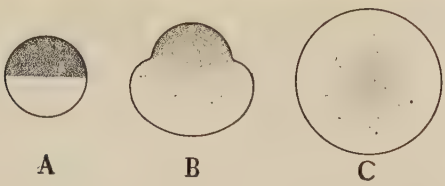
The author followed the course of membrane formation in several species of echinoids available at Misaki and the results will be reported as complementary to Runnström's investigations.

MATERIALS

The regular sea urchins, *Strongylocentrotus pulcherrimus*, *Anthocidaris crassipina*, *Mespilia globulus* and *Pseudocentrotus depressus*, and the sand dollars, *Clypeaster japonicus* and *Peronella lesueuri* are used. The results in *Clypeaster* and *Strongylocentrotus* will mainly be described.

RESULTS

Unfertilized condition. The cortical granules exist in a single layer immediately under the surface of the mature egg. The granules are found in all the above mentioned species. In *Clypeaster*, the cortical granules range in size roughly from 0.5 μ to 1.3 μ, the average being 0.8 μ, but in some batches the average reaches more than 1 μ. The granules of *Strongylocentrotus* average about 0.5 μ. The density of the granules is about 60 in a 10 μ square (= 100 square μ) in *Clypeaster* and 120 in *Strongylocentrotus*.



Text-fig. 1. Diagram illustrating the explosion of a cortical granule, as seen under the phase contrast microscope. A. cortical granule of an unfertilized egg, showing the differentiation into light and dark parts. B. explosion of a cortical granule; the light part swells and the border between the two parts becomes obscure. C. a globule; the granule changes into a large bright globule.

Under the ordinary microscope, the granules of the *Clypeaster* egg appear transparent. Under the phase contrast microscope[1] (dark contrast), the granules are differentiated into light and dark parts (Fig. 6 and Text-fig. 1). However, as the dark part is quite conspicuous, they appear blackish at a casual glance. That such a differentiation is not an optical illusion is indicated by the facts that there is no regularity in the form and location of the two parts in spite of the uniformity in the shape of the granules, and that this differentiation is noticed without exception.

Fertilized condition. In the egg of *Clypeaster*, immediately following the attachment of the fertilizing spermatozoon, the darkish granules suddenly explode and, like grains of popcorn, change into larger whitish ones (dark contrast). If one granule is carefully followed, it can be seen that the part of the granule which is light in the unfertilized condition suddenly swells, and this swollen light part and the more or less unchanged dark part are mixed together. Consequently, the whole granule goes into a larger, light condition (Fig. 2 and Text-fig. 1) which, however, gradually returns to a darkish state. Such a 'changed granule' measures about 1.5 μ in contrast to the former 0.8 μ. This granular explosion begins at the attachment point of the spermatozoon, spreads over the surface of the egg in a wave-like manner, and reaches the opposite side in about 15 seconds, although there is considerable difference in the time of the explosion among neighboring granules.

This change in the granules can be induced by gently pressing and releasing unfertilized *Clypeaster* eggs with a cover glass. The change begins with a short latent period after the release of the pressure, the granules exploding in this case, here and there in a random way. This fact seems to suggest that the granule change is not induced by the direct mechanical force of the pressure, but by an activation caused by the pressure.

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[1] The Bausch and Lomb microscope used in this study was made available by a grant from the American Philosophical Society.

When the eggs are fertilized on a glass slide and observed in a flattened condition, simultaneously with the explosion of each granule, the vitelline membrane separates as a blister corresponding to each exploded granule. If the degree of flattening is less, the vitelline membrane does not form blisters, but is separated evenly from the egg surface. Corresponding to the extent of granule explosion, the membrane elevation starts from the attachment points of the spermatozoon and spreads over the surface. On the rising membrane, the changed granules adhere, forming lens-shaped globules (Fig. 3). Thus it seems that the cortical granules, as they explode, are ejected as globules into the interspace between the vitelline membrane and the plasma membrane, so as to separate these two. The ejected globules adhere to the released membrane, and are elevated with it, gradually becoming flattened (Fig. 4). Within 10 minutes, a smooth and thick fertilization membrane is completed (Fig. 5). The globules can even be observed when the eggs are inseminated in ample sea water.

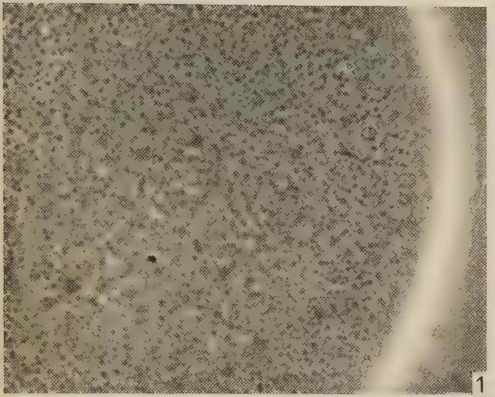
The adhesion of the globules is especially remarkable in *Mespilia* and *Peronella*. In *Strongylocentrotus*, *Anthocidaris* and *Pseudocentrotus*, although the granular explosion is seen, the attached globules cannot be observed.

Formation of rods by treatment with proteolytic enzymes. Unfertilized eggs of *Clypeaster* were immersed in 0.5 per cent trypsin (Kahlbaum) in sea water for 10 minutes or more, or in 0.1 per cent pancreatin (Konishi, Tokyo) for 5 minutes. The enzyme-treated eggs were washed and inseminated.

Runnström (7, 10) and Wicklund (13) report that when membrane elevation is prevented by trypsin treatment, a large number of globules are extruded from the egg surface, a few minutes after insemination. Runnström is of the opinion that these globules contain the cortical granules together with a certain amount of cortical matrix other than the granules.

Clypeaster japonicus.

1. Surface view of an unfertilized egg. The cortical granules are seen as dark spots.
2. Explosion of the cortical granules. Changed cortical granules appear as large light spots. The granules which have not yet exploded appear dark as in 1.
3. Globules adhering to the elevating membrane.
4. Later flattening of the globules.
5. Completed fertilization membrane.
6. Magnified cortical granules showing differentiation into light and dark parts.
7. Fertilization of a trypsin-treated egg. The globules are being extruded from the egg surface. The globules on the upper surface of the egg take irregular shapes because of the pressure of the cover glass.
8. Angular globules. Rods are taking form, without involving the light droplets within the globules.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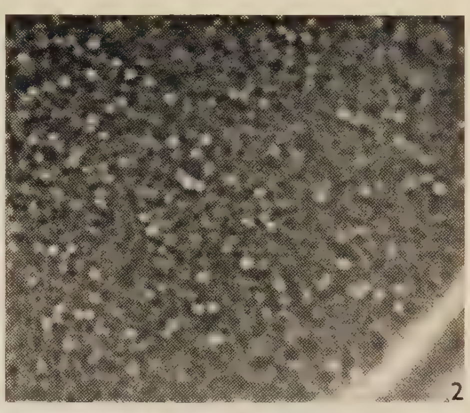
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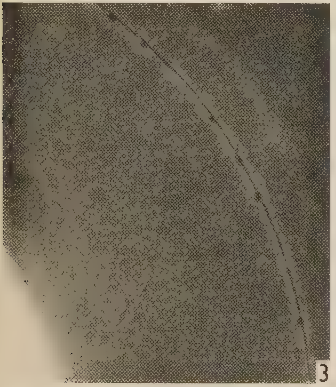
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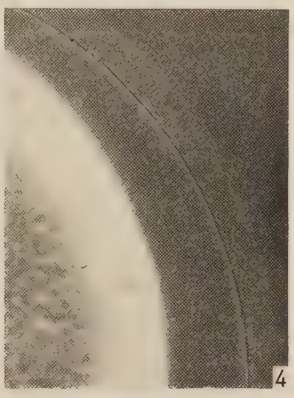
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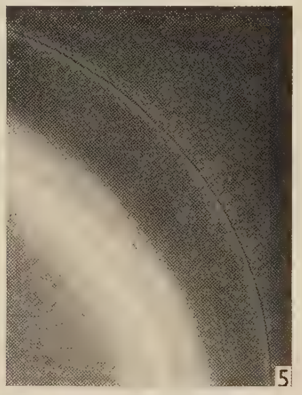
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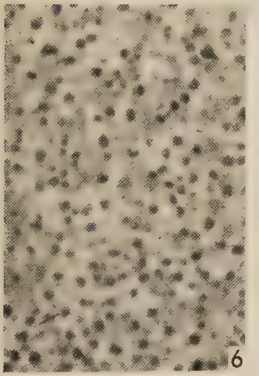
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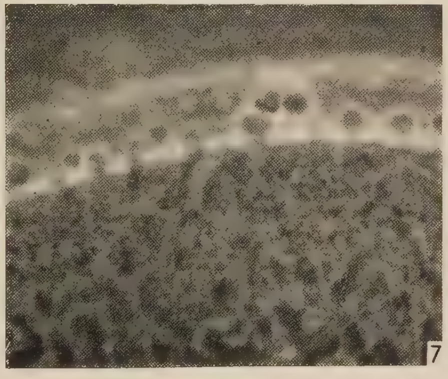
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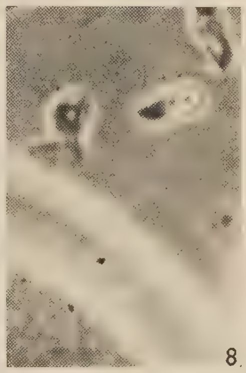
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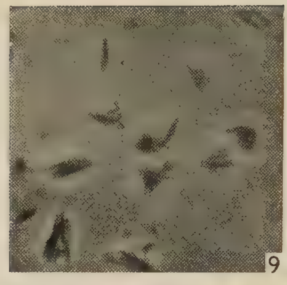
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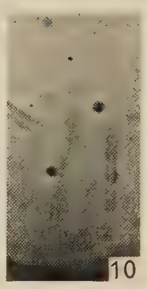
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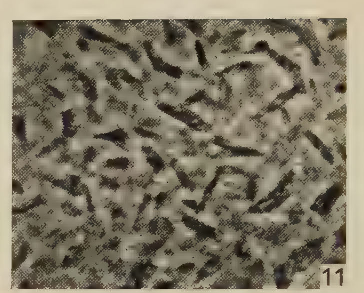
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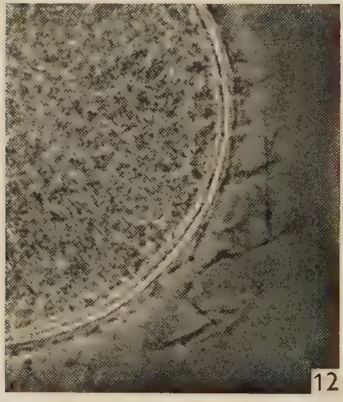
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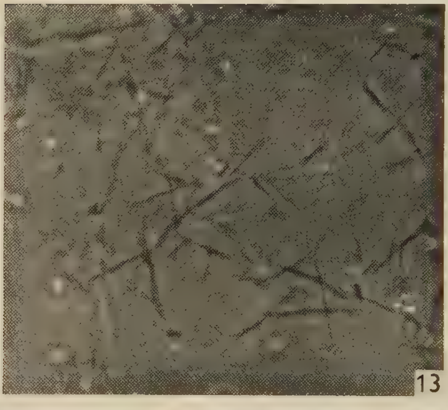
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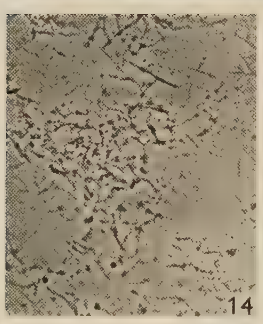
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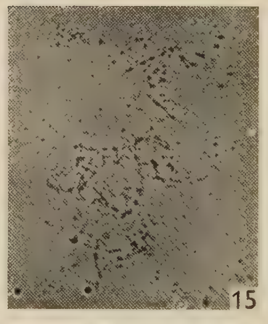
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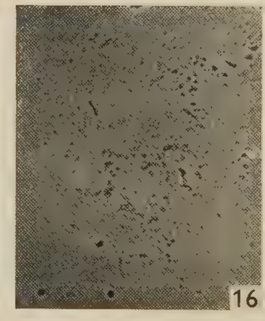
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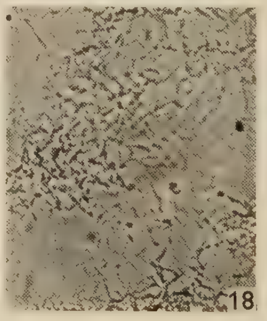
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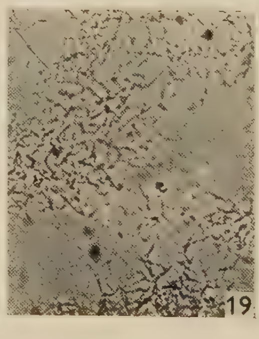
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The author finds it likely that the globules are made of the substance of the cortical granules alone. The basic facts supporting this belief will be presented below. In the extremely transparent egg of *Clypeaster*, a clear-cut phase contrast effect can be obtained even when the microscope is focussed on the upper surface of the egg. Under this condition, a continuous observation of the course of change from the granules to the globules can be made.

In the surface view, the dark cortical granules explode at fertilization and change into larger ones, as was explained above. These changed granules fuse with one another and the contour of the fused mass takes an irregular shape (Fig. 7). Such irregularity is obviously due to the pressure by the cover glass, since similar masses lying on lower levels—consequently not compressed—are all spherical like the globules near the largest optical section of the egg (Fig. 7). The majority of these globules are 2–4 μ in diameter. From the above observation, it can now be understood why the globules extruded from trypsin-treated eggs are larger than those found on the rising membrane of the untreated egg, for the latter adhere to the vitelline membrane separately before they have a chance to fuse together. It must be mentioned that within these globules which now look darkish (dark contrast), often from one to about five bright droplets are found (Text-fig. 2). The process of formation of the droplets was not followed.

With the lapse of time, the globules become angular in contour, and about 5 minutes after insemination, some rod-like precipitates begin to appear within the globules (Fig. 8 and Text-fig. 2). Under the polarization microscope, the rods show a positive birefringence in the direction of the long axis (see also Runnström (7)). The manner of the rod formation is variable

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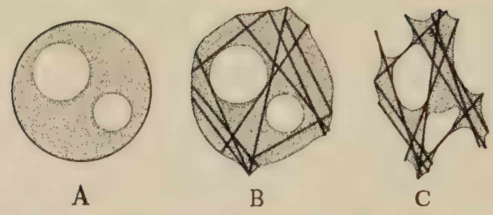
Clypeaster japonicus.

9. Completed rods. Each group is formed from one globule.
10. Two parallel rods in a globule.
11. Rods on the upper surface of an egg.

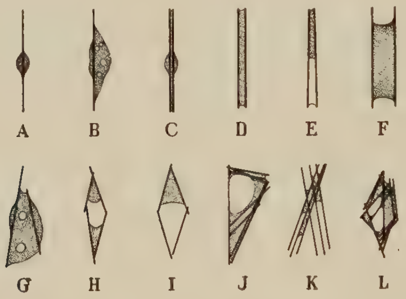
Strongylocentrotus pulcherrimus.

12. Rod formation in trypsin-treated egg. The rods are projecting directly out of the egg surface.
13. Completed rods; these are longer than those of *Clypeaster*.
14. Rods immersed in hatching enzyme solution for 40 minutes.
15. After 2 hours and 40 minutes of immersion.
16. After 3 hours and 20 minutes of immersion.
17. Complete dissolution of the rods after 6 hours and 30 minutes of immersion in hatching enzyme solution.
18. Rods immersed in pancreatin solution for 20 minutes.
19. Rods after 24 hours of immersion in the pancreatin solution, showing no visible change.

(All photographs are taken through a phase contrast microscope.)



Text-fig. 2. Diagrammatic illustration of the process of rod formation in trypsin-treated egg. A. a globule containing two light droplets. B. rods appear in the dark area and the globule becomes angular in contour; the light droplets are not involved in the rod formation. C. the globule, after the completion of the rod formation, showing the remnants of the light droplets in irregular shapes.



Text-fig. 3. Various types of rods; non-dotted areas represent the remnants of the light droplets.

(Text-fig. 3). In some cases, one or two rods are formed within a globule, which elongate to several times the diameter of the globule. When two rods are formed, they often lie parallel to each other (Fig. 10 and Text-fig. 3, C–F). In other cases, more than 10 rods are formed in one globule. These are shorter and lie in random directions (Figs. 9 and 11). The length of the rods usually ranges from 2 μ to 25 μ. No strict correlation is found between the length of the rods and the size of the globules. The globules sometimes form wool-like precipitates. This difference in appearance does not seem to be essential.

When bright droplets exist in the globule, the rods are formed invariably outside these droplets, within the surrounding darkish part (Fig. 8 and Text-fig. 2). There is no coincidence in number between the droplets and the rods.

The rods can also be observed in other species (Fig. 13). In *Strongylocentrotus* eggs, the globules spout out from the egg surface. Frequently, however, the rods project directly from the egg surface without the appearance of the globules (Fig. 12).

The possibility that the rod-formation is an effect of the digestive enzymes employed is rejected on the basis of the fact that similar rods are found to a lesser extent, in the perivitelline space of normally fertilized eggs of many species as will be described below.

Effects of Ca-ion. It is well known that when sea urchin eggs are transferred to a Ca-free medium immediately after insemination, the fertilization membrane remains soft and is elevated higher than normally. The rods which are formed spontaneously in the perivitelline space of normally fertilized eggs mentioned above are missing in Ca-free medium. If the enzyme-treated eggs are handled similarly, the release of the globules cannot be seen, and the rods consequently fail to appear.

On the other hand, the rods, once formed, do not dissolve in a Ca-free medium, whether they are in the perivitelline space or have been freed into the medium by the action of the digestive enzymes. Nor is the hardened fertilization membrane softened. These facts indicate that the rods are the product of an irreversible combination of Ca-ions and the substance of the cortical granules.

Specific effect of hatching enzyme on the rods. It has been known for some time that trypsin and also pancreatin fail to digest the hardened fertilization membrane at room temperature. The digestive enzymes also fail to attack the rod material, which is a Ca compound of the substance of the cortical granules. The statement that trypsin affects the membrane formation, if it is made to work on the unfertilized eggs, should not be confused with the preceding sentence. So far as present experimental evidence goes, the only structure of unfertilized eggs which is attacked by the enzyme is the vitelline membrane.

On the contrary, the hatching enzyme secreted by blastulae can dissolve the fertilization membrane (3). Although Sugawara (12) showed that the vitelline membrane is attacked by the hatching enzyme, the question remains unanswered whether this enzyme attacks the granule substance which is deposited on the vitelline membrane. This situation can be analysed by testing the enzyme action on rods.

The procedure employed is as follows. The hatching enzyme is obtained by Sugawara's method (11) using *Strongylocentrotus* larvae.[1] The strength of the hatching enzyme thus obtained is compared with trypsin or pancreatin by comparing their respective effects on the vitelline membrane of unfertilized eggs. Under optimal conditions, a 10 minute treatment by the hatching enzyme solution corresponds to a 5 minute treatment by 0.5 per cent trypsin solution, or a 1 minute treatment by 0.1 per cent pancreatin solution, in their respective effectiveness in producing the state of the membrane which results in the multinucleated condition. (See Sugawara (12) and also the discussion of this paper.) In short, in its action on the vitelline membrane, the hatching enzyme solution is weaker than trypsin or pancreatin in the concentration used.

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[1] A sufficient number of the larvae is cultivated in a large vessel until a short time before hatching, when they are removed to a small vessel containing an amount of sea water 9 times the volume occupied by the blastulae. After hatching, the blastulae are centrifuged off and the supernatant sea water is used. *Strongylocentrotus* larvae are favorable as material because they can hatch simultaneously in a dense suspension.

In testing these enzymes on the rods, for convenience in handling them, the rods were made to adhere to a cover glass. The procedure is as follows: After insemination of trypsin-treated eggs in a vessel, some of the eggs are placed on a cover glass. The rods fall onto the cover glass as they are formed, and adhere there. After a convenient time, the cover glass is gently rinsed in distilled water and dried, the rods remaining on the glass. The cover glass with the rods is then placed on a hollow slide, the depression of which is filled with the enzyme solution to be tested, care being taken to exclude air bubbles, and the edges of the cover glass are sealed with paraffin.

Successive observations show that rods immersed in trypsin or pancreatin solution remain for over 24 hours without any visible change (Figs. 18 and 19). The rods in the hatching enzyme solution become less and less clearly defined, and after about 5 hours, they are lost from view (Figs. 14–17). In other words, the hatching enzyme is able to specifically affect the rods—derivatives of the cortical granules—in contrast to the inability of trypsin and pancreatin. From this experiment, it can be inferred that the solubility of the completed fertilization membrane in the hatching enzyme and its insolubility in the digestive enzymes are governed by this derivative of the cortical granules.

CONCLUSION

Judging from the facts so far presented, the following conclusions may be drawn. In normal fertilization, when the globules (changed cortical granules) adhere to the vitelline membrane, they combine tightly with the membrane and give rise to a fertilization membrane which shows no structure. (In *Strongylocentrotus pulcherrimus*, there is a vague pattern on the fertilization membrane. This will be the object of a future study.) If the globules are prevented from adhering to the vitelline membrane for some reason, the rods are generally formed within them, regardless of whether they are free in the medium or enclosed in the perivitelline space. Although the derivative of the cortical granules which combines with the membrane cannot be discerned under the microscope, it seems to go through an essentially similar change as in the rod formation. The foregoing opinion is supported by the following facts: Ca-ions are necessary both for the hardening of the fertilization membrane and for the formation of the rods; the optical axis of birefringence of the fertilization membrane and the rods coincides; the time needed for the hardening of the fertilization membrane is about the same as that required for the formation of the rods. The coincidence, between the fertilization membrane and the rods, in solubility in the hatching enzyme and their insolubility in trypsin will also confirm this view. Repeating it, the substance of the rods must be identical with the substance which hardens the fertilization membrane.

DISCUSSION

Moser (4) explained the mechanism of the formation of the fertilization membrane from the standpoint of vacuolization of the cortical granules. In *Clypeaster*, however, it is impossible to find the vacuolization of the cortical granules and their direct transformation into the perivitelline space which Moser assumed would occur. The globules of *Clypeaster* are neither so large nor so many as to account for the perivitelline space by their coalescence. Besides, in *Strongylocentrotus*, the so-called "extra-granular zone," from which the cortical granules seem to have been ejected, can be distinguished on the periphery of fertilized egg (2, 6). If Moser were correct, the part of the egg occupied by the "extra-granular zone" should change into the perivitelline space and go outside the egg.

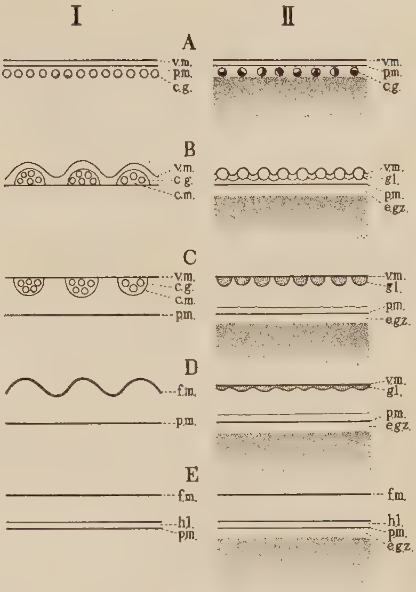
Motomura (5, 6) concluded, mainly from his staining studies with Janus green B, that the cortical granules are secreted between the vitelline membrane and the protoplasmic surface film during the 10 minutes following fertilization. The vitelline membrane is elevated by the colloid osmotic pressure of the exuded granule substance, and hardened by later precipitation of the granule substance on its inner surface. The author's observations in the same species, *Strongylocentrotus pulcherrimus*, reveal that the wave of granule extrusion completes itself within a few minutes after insemination. However, nearly 10 minutes are required for the completion of the membrane hardening. About the mechanism of the formation of the perivitelline space, the author's view opposes that of Motomura. Since the author thinks with Runnström, that the globules do not usually dissolve, they may not be able to contribute to the development of colloid osmotic pressure, although the author does not reject the possibility that some other colloid may escape from the egg at the time of the release of the globules.

Chase (1) observed in *Dendraster* eggs that after sperm entry, blisters containing droplets appeared on the surface of the egg. These droplets disappear as the membrane hardens, and seem to be identical with the globules observed by the author. In spite of Chase's statement, his droplets seem to be attached to the wall of the blisters, for the globules suspended in the perivitelline space always remain, and do not disappear according to my experience. In *Peronella lesueuri*, the globules adhering to the membrane are so conspicuous that at a glance they look as if suspended in the perivitelline space, resembling Chase's figure.

Runnström (7) found rods in the perivitelline space of *Psammechinus miliaris* eggs when these eggs were fertilized after treatment with various reagents. He considered the rods to be derivatives of the cortical granules. In this sense, the present paper is a confirmation of Runnström's results by continuous observation, although there are a few differences in opinion. Runnström believes that in normal fertilization, the extruded globules contain several cortical granules, together with a certain amount of 'cortical matrix.' On adhesion to the vitelline membrane, the globules change their nature as the membrane hardens. The same author (9) finds that the substance derived from the cortical granules constitutes only a minor part of the fertilization membrane, as a result of his calculations which show that the total volume of the cortical granules falls far short of the volume of the completed fertilization membrane. The author's finding, that the size of the cortical granules suddenly increases as they explode, seems to be significant in this connection: that is, the increase in granular diameter may account for the volume difference between the granules and the fertilization membrane.

According to the author's observations, it seems rather doubtful that the substance of the cortical matrix is an essential element of the fertilization membrane. In the normal fertilization of *Clypeaster* eggs, the size of the globules which adhere to the rising membrane is roughly equal to that of the exploded cortical granules. The globules liberated from trypsin-treated eggs are larger than the exploded cortical granules and contain the bright droplets. However, as was explained before, these globules are the coalescence product of the substance of several cortical granules without cortical matrix. The droplets do certainly not correspond to the cortical granules as such. When rods appear within the globules, they are invariably formed within the dark part of the globules, excluding the bright droplets. Textfig. 4 illustrates the controversial points regarding the formation of the fertilization membrane.

Concerning the action of trypsin, Sugawara (12) performed an experi-



Text-fig. 4. Comparison of Runnström's scheme with that of the author, concerning the process of the formation of the fertilization membrane. I. Runnström's scheme (*Arkiv Zool.*, **40**, 1 (1948)) as modified by the author. II. The author's scheme. A. unfertilized condition. B. extrusion of cortical granules. C. adhesion of globules to the membrane. D. further transformation of the fertilization membrane. E. completed fertilization membrane. v.m. vitelline membrane, p.m. plasma membrane, c.g. cortical granule, c.m. cortical matrix, gl. globule, e.g.z. extragranular zone, f.m. fertilization membrane, h.l. hyaline layer.

ment, using *Strongylocentrotus* eggs. He treated unfertilized eggs with trypsin during various intervals. On insemination, as long as the time of immersion was short, a normally hardened membrane was elevated. As the time of treatment became longer, the hardened membrane was less and less elevated, and finally the so-called tight-membrane eggs were obtained. In these tight-membrane eggs, cytoplasmic division was suppressed and multinucleated condition resulted. If the time of treatment was increased, although the membrane failed to be elevated, cleavage became possible once more. This was due to the fact that the membrane remaining on the egg surface became soft enough to allow the change in form of dividing eggs. Repetition of Sugawara's experiment revealed that the stage in which the multinucleated condition was achieved corresponded to the last stage of the enzyme treatment in which the granule substance was retained within the tight-membrane, resulting in its hardening. Further increase of the treatment makes the tight-membrane soft enough so that the granule substance escapes through the membrane, and the rods appear outside of the eggs.

SUMMARY

1. *Strongylocentrotus pulcherrimus, Anthocidaris crassipina, Mespilia globulus, Pseudocentrotus depressus, Clypeaster japonicus,* and *Peronella lesueuri*

were examined concerning the behavior of the cortical granules in the formation of the fertilization membrane.

2. In the eggs of *Clypeaster japonicus,* the cortical granules are differentiated into two optical parts of light and dark, when observed by a phase contrast microscope (dark contrast).

3. Following the attachment of a spermatozoon, the granules explode and change into large globules. The granule explosion begins from the attachment point of the spermatozoon and spreads in a wave-like fashion.

4. With this change, the globules are ejected, and the vitelline membrane is separated from the egg surface.

5. The globules adhere to the rising vitelline membrane, gradually combining with it and giving rise to the fertilization membrane.

6. When the vitelline membrane is weakened by proteolytic enzymes, the globules are squeezed out through the membrane, which remains attached to the egg surface. After several minutes, rod-like precipitates are formed in the globules.

7. Ca-ion is necessary for the hardening of the fertilization membrane and also for the formation of the "rods."

8. It is suggested that the substance responsible for the hardening of the fertilization membrane is the same as the substance of the rods.

9. The hatching enzyme of *Strongylocentrotus pulcherrimus* acts specifically on this substance.

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THE CORTICAL PROPAGATION OF THE ACTIVATION IMPULSE IN THE SEA URCHIN EGG

JOHN RUNNSTRÖM and G. KRISZAT

The Wenner-Gren Institute, University of Stockholm, Sweden

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THE conception of a wave propagation of the impulse caused by the attachment of the spermatozoon to the egg surface has been discussed repeatedly, see Just (5). In a very meritorious paper Rothschild and Swann (7) recently pointed out that the propagation of this impulse must not necessarily be very rapid. As a criterion of the propagation of the impulse they utilized the colour change of the egg surface which can be observed with dark ground illumination, see Runnström (9, 10, 13). The conduction time of this change was found to be 20–30 seconds in *Psammechinus miliaris* (7). This conduction rate seems to be sufficient to protect against polyspermy which indicates that only a fraction of the collisions between spermatozoa and eggs is successful (7, 8).

Rothschild (6) has discussed the possibility that the cortical changes are due to the diffusion of a substance derived directly or indirectly from the sperm head. The diffusion may be "intracortical" or "intracellular." Rothschild favours the latter alternative.

Below, certain results will be reported which speak rather in favour of a cortical conduction than a conduction through the interior cytoplasm.

Moreover, the present writers prefer to assume that the propagation of the impulse in fertilization depends on a chain reaction rather than on pure diffusion. This reaction may be started by a primary local reaction caused directly by the attached spermatozoon.

The primary reaction may be induced already in the oocyte. As is well known this responds to insemination by formation of hyaline protrusions (for references see 6 and 13). The oocyte, however, is still incapable of responding to the primary reaction by setting up a chain reaction. Therefore a great number of spermatozoa enter the oocyte in which they do not develop further. Under certain conditions a rather similar state can be observed also in the mature egg.

METHODS

The observations presented below are based on the experience that eggs of *Psammechinus miliaris* often adhere to the bottom of the glass dishes when deprived of their jelly coats (15). These were removed by subjecting the eggs for 10–15 seconds to acid sea water (pH 3.5) in a glass cylinder. The acid treatment was rapidly interrupted by adding a tenfold volume of sea water of pH 8.0. The eggs were stirred at the same time by rotation of the cylinder. After several minutes the eggs were allowed to settle to the bottom. They did not adhere to the glass of the cylinders used. Upon transfer of the eggs to smaller dishes used in most of the experiments attachment to the bottom of the glass usually occurred. The same result, rather inconvenient for ordinary work, was obtained even when the dishes were made of glass from Schott and Gen. ("Jena-glass"). Addition of two drops of 0.1 per cent serum albumin in sea water to 2 ml suspension of eggs prevents their attachment (15).

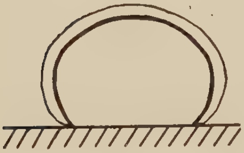


Fig. 1 a.

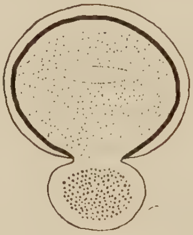


Fig. 1 b.

Fig. 1 a. Egg attached at a glass surface. Membrane formation only from the free surface.
Fig. 1 b. Egg removed from its attachment to glass with an unfertilized protruding lobe.

The attached eggs were fertilized upon insemination and a membrane elevation occurred, fig. 1 a. For many years it was observed, however, that the part adhering to the glass surface failed to form a membrane. By blowing with a pipette it was possible to remove the eggs from their attachment to the glass. This operation was carried out at different time intervals after the insemination, usually after 30 minutes, but in certain cases only after 90 or 120 minutes.

In general the entire egg is removed by blowing. The greater part of its surface is covered by a membrane, as shown in the diagrammatic fig. 1 b. One part of the egg cytoplasm, however, forms a hernia-like protrusion, which may be separated from the main part of the egg by a constriction.

Sometimes one does not succeed in detaching the part of the egg adhering to the glass. The main part is pulled off, but the smaller protruding part remains attached. Finally this latter may, however, also be detached as a consequence of the blowing by the pipette. The egg is thus separated into one larger and one smaller fragment.

A great number of different cases offered themselves to the observer in the present experiments. The following descriptions are based on combined inspection of mass cultures and isolation of single specimens. The frequency of the different types mentioned below has not been exactly determined. Since the main variations depend on fortuitous circumstances, such determinations would be of little interest.

RESULTS

Through the method mentioned eggs of the type shown in fig. 1 b were isolated. As already stated, the larger part of the egg was covered by a normal fertilization membrane. This part assumed an almost spherical form, owing to the increase in elasticity and rigidity which occurs in the cortical layer subsequent to fertilization (11, 12). The projecting part of the egg had no membrane. No nucleus was visible in the interior; it may thus be inferred that the projecting part remained unfertilized. Its hernia-like form is probably due to the pressure exerted by the elastic cortical layer of the larger fertilized part of the egg.

The following additional criteria concerning the different states of the main and the projecting part of the egg were also fulfilled in the cases referred to.

1) In *Psammechinus miliaris* the eggs have clearly a less homogenous appearance before than after fertilization (9, 13). This impression is sometimes accentuated by the presence of rather coarse granules within the cytoplasm of the unfertilized egg. Upon fertilization the ground substance becomes more homogenous and the granules are dispersed.

2) Subsequent to fertilization the eggs exhibit angular plasmolysis upon immersion in a hypertonic medium (2 ml sea water +2.5 ml·2.5 N NaCl), see (11).

In a case like that represented in fig. 1 b the main part of the egg showed the homogenous appearance characteristic of the fertilized state, whereas in the protrusion the course granules were clearly visible. Upon immersion in a hypertonic medium, e.g. 30 minutes after insemination, the main part of the egg shrank rapidly assuming an angular form. The hyaline layer became clearly visible as a rather broad seam. The protruding part on the other hand shrank more slowly showing the characteristic wrinkled or smooth shape of its surface. The absence of the hyaline layer was obvious.

Cases like that reported were rather rare, as a secondary fertilization of the protruding part usually occurred upon detachment of the egg from the glass. Initially new sperm was added at that moment, but this proved not necessary, as the first added portion of sperm remained active for a sufficient period of time. The course of events following upon fertilization of the protruding part depended evidently on the degree of injury that its surface had suffered when the egg was detached from the glass.

It is important to state that in a number of well defined cases a normal membrane formation and later a cleavage took place, fig. 2. This was delayed in comparison with that of the main part of the egg. The developing fragment gradually separated from the main embryo. Small swimming blastulae were observed, but a gastrulation of these never occurred. Sometimes small cleaved fragments also remained attached to the main embryo.

These cases were, however, the rarest. Even if the development of the fragments proceeded, the cleavage of the cells often proved to be incomplete. Under these circumstances the blastocoel arose in a secondary way by fusion of a number of vacuoles arising in a syncytium. The process has not been studied in detail, but the phenomenon agrees with that already described by Godlewski (2).

With respect to the development of the protruding fragments a whole gamut of different pathological cases was displayed.

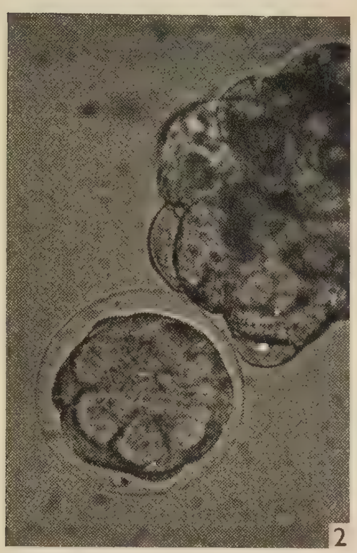
1) No fertilization membrane was formed, or the membrane did not harden. In the latter case it was soon broken up. Cleavage occurred, fig. 3.

2) A fertilization membrane was formed, but no cleavage took place, fig. 4.

3) No fertilization membrane appeared and no cleavage occurred, but a number of spermatozoa were seen to penetrate into the egg, fig. 5.

Some further details should be mentioned concerning case 3. The formation of reception cones was followed by continued observations of the egg. Several of these cones could be formed almost simultaneously. They had the shape of rather large lobes. In one particular case two such lobes were withdrawn after 8 minutes. Immediately afterwards a number of much thinner protrusions appeared with a spoon-like enlargement at their free ends. Again these protrusions were retracted. A new protrusion could now appear at a different site. In many cases direct observations on living material revealed that the formation of a lobe or protrusion corresponds to the penetration of a spermatozoon. In this way a great number of spermatozoa could be trapped in a small egg fragment. Fig. 5 shows a living egg fragment in the interior of which a number of nuclei could be discerned. No radiation was seen in fragments with lobe formation, but the nuclei swelled and also seemed able to grow (or to coalesce).

The conditions in the fragments described under 3, see above, are very similar to those prevailing in oocytes. A spermatozoon causes a local reaction which brings about the formation of a protrusion, but this local reaction is not propagated. Even the form of the protrusion recalls in a striking manner those observed upon insemination of oocytes. It occurred for example that a broader lobe appeared primarily in a fragment. Secondarily, this was split up into a number of fiber-like structures. The same was the case with the pro-



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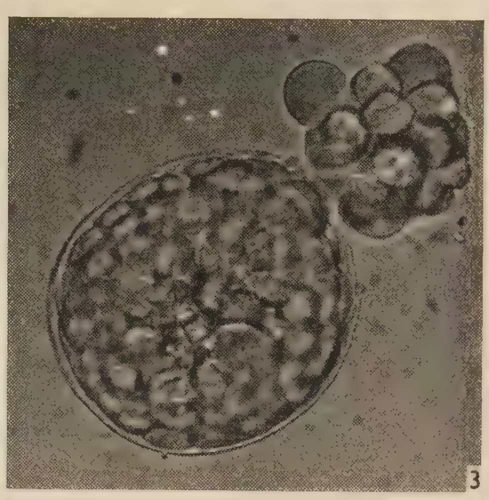

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Fig. 2. Fragment from a previously attached egg. Normal membrane formed.
Fig. 3. Previously attached egg. Fragment with the membrane thrown off.

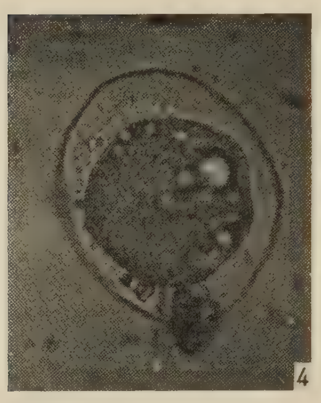
trusions found in inseminated oocytes, and in normal reception cones as well.

It is thus evident that a mature egg or at least a fragment of an egg may be converted into a state similar to that prevailing in the oocyte. This must be due to the partial removal or damage of the cortical layer.

The spermatozoa penetrating into a fragment which gives only local reactions do not cause any radiation in the interior of the cytoplasm. This again is analogous to what happens in the oocyte upon penetration of spermatozoa. On the other hand, the incomplete polyspermic fertilization causes the same changes in the collodial state of the cytoplasm as does normal fertilization. The ground cytoplasm becomes more homogenous and the coarse granules are dispersed.

In certain cases the main part of the egg behaved abnormally, e.g. the formation of segmentation furrows was incomplete or failed altogether. Despite this, the protruding part could be fertilized and undergo segmentation.

In the cases referred to above, the protruding fragment became completely or almost completely separated from the main part of the egg upon fertilization. In other cases, however, a broader connection remained between the two parts of the egg. Under these circumstances the protruding part was gradually nucleated from the main part and incorporated with the embryo. This process has, however, not been followed in detail.



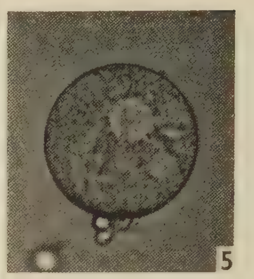


Fig. 4. Fragment from a previously attached egg. Membrane elevated but no cleavage.
Fig. 5. Fragment from a previously attached egg. No membrane and no cleavage, polyspermy.

DISCUSSION

The results reported above may be summarized in the following way:

1) The propagation of the impulse caused by the attachment of the spermatozoon is inhibited by the attachment of the egg surface to glass. The underlying cytoplasm remains "unfertilized" as well.

2) If the egg is detached from the glass, the unfertilized part of the egg appears as a hernia-like protrusion which may now be fertilized.

3) If the cortical layer remains intact, segmentation of the protruding egg fragment is possible. Development beyond blastula stage was not observed.

4) If, on the other hand, the cortical layer has suffered a certain damage, the fragments respond only with local reaction to the attachment of spermatozoa, and polyspermy occurs.

5) In eggs with damaged cortical layer no radiation develops around the sperm nuclei and no segmentation occurs.

The data sub 1) and 2) indicate that the impulse caused by the attachment of the spermatozoon propagates primarily through the cortex.

The fragments with damaged cortical layer emphasize the contrast between the primary reaction caused by the attachment of the spermatozoon and the propagation of this effect. As pointed out above, the injury of the cortical layer brings about a state reminiscent of that prevailing in the oocyte.

It seems rather probable that the primary impulse is propagated as a chain reaction. The primary impulse activates adjacent units (macromolecules or macromolecular complexes). These in their turn give off impulses and so on. The attachment to glass might block the propagation of these impulses by fixing the macromolecules of the cortex in their original position, whereas activation brings about their flowing and partial reorientation (for further discussion, see (14)).

If the interesting curve, published by Rothschild and Swann (7, text-fig. 1) is replotted, so that changed surface area replaces angle subtended, the plateau on the curve becomes less conspicuous without disappearing altogether. As only one curve of this kind is available, it would be premature to speculate about the nature of the plateau. It may only be pointed out that the surface of the mature sea urchin egg is not uniform in its properties. This follows from Gustafson's (3) experiments, in which certain regular indentations arose on the egg surface subsequent to exposure to certain amino acids. Gustafson's results open the possibility that different parts of the egg surface are less susceptible to sperm attachment than others, and also that the rate of propagation may be different in different directions within the surface.

In joint work with C. Harding, the senior writer confirmed in a qualitative way that the propagation of the colour change accelerates towards the end of the process. This acceleration is rather well in keeping with the views presented here. When more than half the egg surface area has undergone the colour change, its frontier advances from regions containing a relatively larger amount of activating units towards regions with fewer and fewer activateable units per circumference. The impulses hitting those units therefore become relatively more frequent.

Rothschild (6) has submitted the present problem to mathematical treatment, in order to decide whether intracellular or intracortical diffusion are to be considered as the mechanism of the propagation of the impulse. The present writers cannot see, however, that the comparison between theoretical and experimental curves presented in Rothschild's (6) fig. 1 and fig. 2 permit any choice between the two alternative hypotheses.

The assumption of a chain reaction presented above is in agreement with the well supported view that the main process of activation resides in the egg, whereas the sperm attachment mainly acts as a trigger mechanism. In parthenogenesis this mechanism is replaced by the activating agent. Runnström (9) observed that upon immersion of unfertilized eggs in hypotonic medium, the break-down of the luminous layer which is visible in darkfield, starts at one point and then travels around the surface. It is not known whether the same applies to real parthenogenesis. In the observations just quoted the eggs cytolyzed. In fertilized eggs subjected to diluted sea water, the luminous layer faded away uniformly over the whole surface of the egg (9).

In micrurgical work Chambers (1) proved that cortical fragments of the egg are fertilizable, whereas the interior cytoplasm is irresponsive to the action of the spermatozoon. This again underlines the master role of the cortex in the activation. As follows from dark field observations (11, 13), the cortical changes are soon followed, however, by interior changes. The data presented above exclude that mere diffusion of low molecular substances is instrumental in bringing about these changes. They are rather initiated and propagated by an interaction of macromolecules which spreads from the cortex to the interior of the egg.

It is obvious that results such as those reported above may have some bearing on teratology. A blocking of the activation impulse may give rise to parts which fail to be integrated into a harmonious embryo.

SUMMARY

On the basis of observations carried out on eggs attached to a glass surface, it was inferred that the impulse caused by the sperm attachment primarily spreads in the cortical layer.

The primary action of the spermatozoon is considered to be propagated by a chain reaction, in which activateable units present in the cortex are concerned.

The factual observations are summarized in points 1–5 under "Discussion," see p. 424.

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DEGRADATION OF STARCH, HYDROXIETHYL CELLULOSE ETHER AND CHITOSAN BY ENZYMES IN SPERMATOZOA AND SPERM FLUID FROM *PSAMMECHINUS* AND *MODIOLA*

ESKIL HULTIN and GUNNAR LUNDBLAD

Biochemical Institute and the Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden[1]

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A more detailed knowledge concerning the enzymes present in the spermatozoa is of prime importance in the study of the physiology of fertilization. McClean and Rowlands (9) have demonstrated the presence of hyaluronidase in the spermatozoa of the rabbit. The dispersion of the cumulus cells is probably due to this enzyme. The presence of a lytic agent in the spermatozoa of two snails, *Megathura crenulata* and *Haliotis crucherodi* has been demonstrated by Tyler (12) whose work was continued by Krauss (6). Berg (1) found the lytic sperm factor also in *Mytilus edulis*. The lytic sperm substance seems to have a protein nature, but it has not been characterized in a more detailed way. Monroy and Ruffo (10) and Lundblad and Monroy (8) have found the extracts of sea urchin sperm to split the jelly coat surrounding the egg and, to a certain extent, also hyaluronic acid. Vasseur (13) also demonstrated the presence of a jelly splitting enzyme on the surface of the sea urchin spermatozoa. As the jelly is constituted to 80 per cent of polysaccharides, the enzyme involved may have a carbohydrase nature. Further studies are necessary, however, in order to elucidate the nature of the enzymes under discussion.

In the summer 1950 one of the writers carried out studies on enzymes splitting chitosane (deacetylated chitin). In the course of these studies some evidence was also found of the presence of a chitosane-splitting enzyme in the male gonads of the mussel *Modiola modiolus*. These studies were taken up again in 1951 with a view to decide whether the chitosane-splitting activity resides in the spermatozoa or in the seminal fluid or perhaps in both. The research was extended also to the sea urchin *Psammechinus miliaris*. Furthermore, some more polysaccharides were tested as substrates.

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[1] The experiments were made at Kristinebergs Zoological Station, Fiskebäckskil, Sweden.

MATERIAL AND METHODS

Extracts of spermatozoa. The spermatozoa of ripe specimens of the sea urchin *Psammechinus miliaris* and of the mussel *Modiola modiolus* were obtained by dissection of the gonads. The semen given off from the gonads was pressed through bolting silk, diluted with sea water and centrifuged at 6 000 rpm for 5 minutes in an angle centrifuge. The sedimented spermatozoa were resuspended in sea water and centrifuged off, the procedure being repeated once more. The spermatozoa retained a good motility after this treatment, as checked under the microscope. The spermatozoa were finally suspended in distilled water for cytolysis, frozen and ground in the frozen state in a chilled mortar. The ice powder thus obtained was thawed out, the fluid kept at +3° C over night and then centrifuged. The residue was discarded and the supernatant stored in the refrigerator for activity determinations.

Substrates. The stock substrate solutions were made up in the following way. Starch solution—1 per cent—was made by boiling a suspension of native potato starch in water for some minutes. The pH of the solution was then adjusted to 7.0–7.5 by adding disodium phosphate, whereupon the solution was autoclaved in an ordinary Flex Seal pressure cooker for 15 minutes. It is necessary to autoclave starch solution if viscosimeters with narrow tubes are used; other methods (4, 5) do not achieve complete dissolution of the starch.

Hydroxiethyl cellulose ether—introduced by Zeise (14) for the determination of cellulases—was prepared by treating filter paper with an alkaline solution of ethylene oxide in acetone. The mixture was neutralized and dialysed against water. The solution was boiled for a few minutes and centrifuged, and the insoluble matter was discarded. A 0.4 per cent solution was used for the experiments.

The chitosane solution was made from a chitosane preparation, obtained from prawn shells which mainly consist of calcium carbonate and chitin. The calcium carbonate was extracted with dilute hydrochloric acid, and the chitin was deacetylated by boiling with sodium hydroxide solution (2). The chitosane thus obtained was dissolved in hydrochloric acid, precipitated with acetone and dried. A 0.35 per cent solution was made.

Buffer solutions. For buffer solutions acetate and acetic acid were used for pH below 5.5, phosphates for pH between 5.5 and 7.5, veronal and hydrochloric acid for pH between 7.5 and 8.8 and ammonium chloride and potassium hydroxide for pH above 8.8. All buffer solutions contained sodium chloride. For amylase and cellulase activity determinations buffer solutions of ionic strength $\mu = 0.1$ were used. Buffers containing phosphate were 0.04–0.06 M in sodium chloride. Buffer solutions used together with chitosane had the ionic strength $\mu = 0.7$, giving an ionic strength of $\mu = 0.18$ in the reaction mixtures.

Activity determinations. The degrading effect of the spermatozoa enzymes on starch, hydroxiethyl cellulose ether and chitosane were determined viscosimetrically (3, 4, 5, 7). One ml extract, one ml buffer solution and two ml substrate solution were mixed and two ml thereof was transferred to an Oswald viscosimeter, whose flow time was about 40 seconds for water. As the viscosimeters available were calibrated for the determination of proteolytic enzymes with gelatin at 35.5° C (11), the determinations were carried out at that temperature, although it is precariously high.

TABLE I

Washing of *Psammechinus* spermatozoa. The cellulase activity.

	pH	mμA/ml	mg N/ml	mμA/mg N
1. Supernatant	6.6	0.19	0.060	3.2
2. Supernatant	6.6	0.02	0.055	0.4
3. Supernatant	6.6	0.00	0.047	—
Spermatozoa extract ...	6.8	0.14	0.46	0.30

The activity calculations were made according to the formula (3, 5)

$$\mu A = c^2 \cdot \mathrm{d}\,(1/\eta_{sp})/\mathrm{d}t \cdot 10^6,$$

where μA = the activity of the enzyme *in the reaction mixture*, c = the concentration of the substrate *in the reaction mixture* (gram substrate per gram solution), η_{sp} = the specific viscosity, and t = the time in seconds.

The equation defines a unit μA for enzymic activity; $\mathrm{m}\mu A = \mu A \cdot 10^{-3}$.

The activity of the *enzyme solution* was calculated from the enzyme activity in the *reaction mixture* by multiplying with the dilution factor.

EXPERIMENTAL

Carbohydrases in Psammechinus miliaris spermatoza. It is not unusual that enzymic activity is attributed to spermatozoa, although it is not proved whether the activity derives from the spermatozoa, themselves or from the seminal fluid. As it was considered important to establish the presence of carbohydrases in the spermatozoa, these were washed several times, and as a control, the enzymic activity was measured of the supernatants which were obtained after the spinning down of the spermatozoa. The results from an experimental series with hydroxiethyl cellulose ether as substrate is given in Table I. The results show that both the seminal fluid and the spermatozoa have enzymic activity and that the spermatozoa were washed sufficiently to remove the medium. It follows also from the experiments that the carbohydrases in the spermatozoa do not easily leak out into the surrounding medium.

The capacity of the extract from spermatozoa to degrade hydroxiethyl cellulose ether was determined at various pH. The results given in Fig. 1 indicate that the optimum activity is at pH 6. Mixtures of enzyme solution, and some different buffer solutions with pH between 5 and 7 were heated to 45° C for ½ hour in an experimental series before the hydroxiethyl

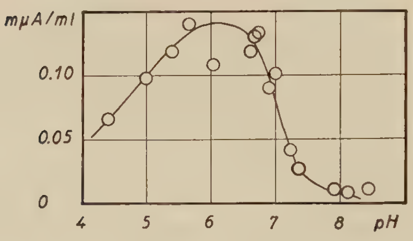

Fig. 1. The activity of cellulase in *Psammechinus* spermatozoa versus pH.

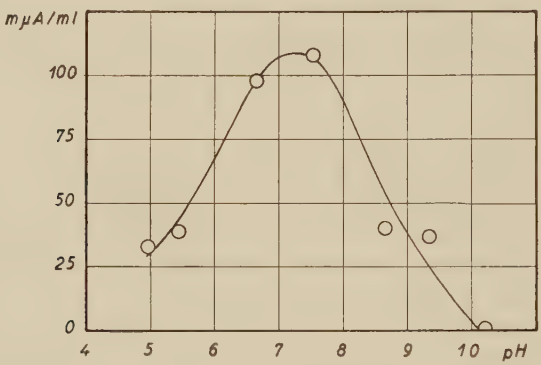

Fig. 2. The activity of amylase in *Psammechinus* spermatozoa versus pH.

cellulose ether solution was added. The activity of the enzyme had diminished to about $\frac{1}{2}$ of its original value. The nitrogen content of the enzyme solution was 0.46 mg N/ml.

A few test experiments made using chitosane for substrate, indicated that the spermatozoa extract had a perceptible power to depolymerize this polysaccharide.

The amylase activity of spermatozoa preparations was measured at various pH, using acetate, phosphate, ammonium, and veronal buffer solutions containing sodium chloride. The ionic strength in the reaction mixture was always $\mu = 0.025$. Fig. 2 gives the result from a series, indicating that the optimum activity is at pH about 7. However, it should be noticed that various buffer solutions with different amounts of sodium chloride were used. The amylase activity of the spermatozoa extract (fig. 2) at 35.5° C and optimum pH was 0.11 $\mu A^{35.5°}$/ml. The nitrogen content was 0.49 mg N/ml. Thus the amylase activity in relation to the nitrogen content was 0.22 $\mu A^{35.5°}$/mg N or, using a conversion factor previously determined (5), $22 \cdot 10^{-6}$ milliequivalents reducing sugar/min · mg N.

TABLE II

Washing of *Modiola* spermatozoa. The cellulase activity.

	pH	mμA/ml	mg N/ml	mμA/mg N
1. Supernatant	5.9	4.6	0.36	13
2. Supernatant	5.9	1.9	0.058	33
3. Supernatant	5.9	0.18	0.037	4.9
Sperma extract	6.3	0.28	0.026	11

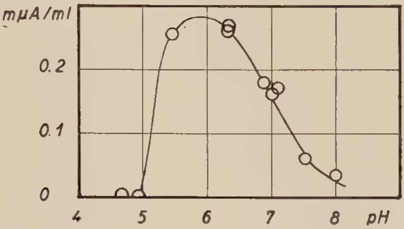

Fig. 3. The activity of cellulase in *Modiola* spermatozoa versus pH.

TABLE III

Modiola spermatozoa. The breakdown of chitosane. Phosphate buffer containing sodium chloride. pH = 6.1; $\mu = 0.18$.

	mμA/ml	mg N/ml	mμA/mg N
1. Supernatant	0.040	0.031	1.3
Spermatozoa extract	0.043	0.14	0.31
Control with boiled extract.............	0.007	0.14	0.05

Carbohydrases in Modiola modiolus. The spermatozoa were washed several times as described to remove their natural suspending medium and the purification was checked by measuring the power of the supernatants to break down hydroxiethyl cellulose ether. The results are given in Table II and show that both the spermatozoa and their natural medium have cellulase activity.

The power of the *Modiola* spermatozoa extract to depolymerize hydroxiethyl cellulose was measured in various buffer solutions. The optimum pH was found to be 6 as shown in Fig. 3. The nitrogen content of the extract was 0.026 mg N/ml.

Chitosane was used as substrate in a few tests with another *Modiola* sperm extract (Table III). In several cases, the reaction mixture gave a precipitate and also other difficulties were encountered. However, the experiments demonstrated the ability of the extract of washed spermatozoa to depolymerize chitosane.

CONCLUSION

It has been demonstrated by the experiments reported here that various carbohydrases are present not only in the seminal plasma but also in the spermatozoa. At least two distinct enzymes are present: amylase and cellulase. The ability of the extract of spermatozoa to depolymerize chitosane may be ascribed to a third enzyme or tentatively looked upon as an effect of a cellulase of low specificity. It is of physiological interest to notice that carbohydrases in the semen are available both in the spermatozoa and in the suspending medium. The carbohydrases of the spermatozoa are not easily given off to the medium from the intact cells, but are rapidly dissolved after the cell membranes of the spermatozoa have been ruptured.

SUMMARY

The semen of *Psammechinus* and *Modiola* contains cellulase, and has also the ability of depolymerizing chitosane. The semen of *Psammechinus* also contains amylase, whereas *Modiola* was not tested in this respect. The enzymes are present both in the spermatozoa and in the suspending medium. The optimum pH for activity is for amylase about 7 and for cellulase about 6.

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ELECTRON MICROSCOPE STUDY OF CULTURED NERVOUS TISSUE[1]

E. DE ROBERTIS and J. R. SOTELO

Departamento de Ultraestructura Celular, Instituto de Investigación de Ciencias Biológicas, Montevideo, Uruguay

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Since the classical work of Harrison (18) a large number of papers has been dedicated to the study of the morphology and properties of cultured nervous tissue with the optical microscope. For references see Levi (23, 22), Levi and Meyer (25), Fell (16). However, no ultrastructural analysis with the electron microscope has been yet attempted on this material.

While most tissue cells developing in an unorganized type of growth undergo a process of dedifferentiation and lose most of the specific characteristics of the tissue, nerve cells continue their differentiation *in vitro*, and may undergo the typical changes observed in embryonic development (4). These changes refer not only to the shape of the cell, but mainly to the development of the specific fibrillar substance of the neurone which is found in the nerve cell body and in all nerve expansions. This specific substance has been observed *in vivo* in the nerve fibers and cells of invertebrates (2, 6, 20) and in the nerve cells and fibers cultured *in vitro* (37, 24). After fixation and staining, this material constitutes the neurofibrils of classical Histology.

According to Levi (23) "the neurofibrils visible with the optical microscope, continue insensibly into an *analogous but ultramicroscopic structure characterized by the longitudinal orientation of the micels of the neurofibrillar substance.*"

Recently, the electron microscope analysis of nerves from invertebrates and vertebrates fragmented by different methods, led one of us to identify a new fibrous component to which we gave the descriptive name of "neurotubule" (12).

Neurotubules are labile structures which undergo disintegration during the degeneration process of the nerve (8, 13). Neurotubules disintegrate in fresh mammalian nerves and show a tendency to break into a vesicular

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[1] This study was carried out with grants of the Rockefeller Foundation to this Department.

material (12). The study of nerves experimentally infected with neurotropic virus transmitted through the axon (poliomyelitis and B virus) shows the presence of particles in intimate relation with the neurotubules (14, 7).

Although fragmentation techniques are not well suited to establish the localization of this component, the available data supported the view that it was an axonic constituent. Recently, this interpretation has been contradicted by several authors. By using sectioning techniques they found filaments of 100 to 200 Å, with nodose contour in thin sections of the axon. According to some of them, the filaments are arranged in a kind of reticulum (27, 35, 34), while according to others they are parallel to the axis (1, 15).

We have recently (9, 10) reinvestigated the problem of the localization of neurotubules and have shown experimentally that the embedding and ultrasectioning techniques introduce a great deal of artefact leading, very often, to the retraction and disintegration of neurotubules into thin filaments. However, when artefacts are at a minimum, the axon appears to be composed of compact parallel bundles of neurotubules. Furthermore, in nerve fibers grown *in vitro* and observed under the electron microscope, the neurotubules are observed in a tight parallel packing (11).

The problem of localization of neurotubules will not be discussed further (see 10). In the present work we try to give a general description of the observations made with the electron microscope in embryonic nerve tissue cultured *in vitro*, and particularly to analyse at a submicroscopic level the organization and fibrogenesis of the specific fibrillar substance of the developing neurone.

TISSUE CULTURE TECHNIQUE AND PREPARATION OF SPECIMENS FOR THE ELECTRON MICROSCOPE

Nervous tissue from the chick embryo develops *in vitro* in contact with a fibrin clot, with or without the addition of embryo extract. Under these conditions the differentiation of nerve cells continues and nerve fibers grow in the surrounding medium. Unfortunately the presence of fibrin interferes considerably with electron microscope observation. All tentatives made to obtain growth of nerve fibers in a liquid, such as Tyrode, blood serum, serum plus embryo extract, failed entirely. For this reason, at the beginning of these experiments we used very thin and diluted layers of plasma. Some results were obtained with chick plasma diluted 1/10 to 1/20 in Tyrode. In such a medium growth of nerve expansion is meagre, stopping in general after 24 hours followed by degeneration. In this first series of experiments pieces of nerve tissue mainly from the brain stem and rhomboencephale of 6–9 days old chick embryo were cultured in hanging drops on coverglasses coated with "formvar" as described by Porter *et al.* (31). After 18 to 24 hours incubation the cultures were washed in warm Tyrode, fixed with osmium vapors for 1 to 5 minutes and then washed in distilled water.[1]

We have recently developed a technique by means of which these difficulties are partially obviated. The nerve explants can be grown in thick layers or more concentrated plasma, being also possible to add embryo extracts. Films are made by depositing one drop of a 2 per cent solution of parlodion in amyl acetate on a sterile Ringer solution. The pieces of tissue are then deposited on the film and surrounded by a drop of plasma diluted 1/4 or 1/6 in Tyrode, with or without addition of embryo extract. The film plus the culture are picked up with a coverglass which is previously deposited on top of the film. Nerve axons grow abundantly inside the fibrin clot but some of them become attached to the parlodion film which covers the explant. On stripping the film most of the nerve fibers and cells stay within the fibrin clot, while some remain attached to the film.

By using this technique we prepared explants from the rhomboencephale and mesencephale of 3 to 14 days old chick embryos. The cultures were incubated for 18 to 48 hours and fixed with osmium vapors[2] when the growth of nerve fibers was considered convenient. In cultures of young embryos (3 to 5 days) only few fibers develop and the explant becomes surrounded by a ring of cells, some of them with the appearance of neuroblasts, while others look similar to indifferentiated fibroblast-like cells. The best growth of nerve expansions within the surrounding medium was obtained with explants of 6 to 8 days old embryos. In these cultures only a few typical neuroblasts migrate and may become attached to the parlodion film. Some of the cells within the explant come also in contact with the parlodion film and can be stripped and observed with the electron microscope. In older cultures (12 to 14 days) migration of cells is very scanty. Nevertheless, typical multipolar neuroblasts or membranous fragments of nerve cells may become attached to the parlodion film.

In a few experiments staining with methylene blue was used on the living cultures. The vital dye was employed diluted (1/20 000 in Tyrode at 38° C for 10 to 30 minutes), Staining of the nerve fibers was controlled under the optical microscope and upon development of a slight blue color this was stopped by washing in Tyrode and by fixating in osmium tetroxide vapors.

The formvar or parlodion films were stripped under the binocular widefield microscope, transferred upon the screens and kept upon phosphorus pentoxide before observation with an RCA EMU electron microscope. Most screens were observed directly without further treatment, but in some cases they were shadowed with chromium or palladium at an angle of 11°. Electron micrographs were taken at magnifications ranging between 1 000 and 9 000 ×.

RESULTS

A general microscopical description of the nervous tissue cultured *in vitro* is beyond the scope of this work. Within the range of the optical microscope this material has been thoroughly investigated (18, 21, 22, 23, 39) and our observations agree with previous works, mainly those of Levi and co-workers who have described in detail cultures of the same material and in more or less similar conditions to ours. We shall stress here only those morphological details which are beyond the resolving power of the optical microscope. Within limits we shall try to bridge the gap between ultrastructural organization and structures recognized with the microsocpe as generally accepted in the literature. In order to be objective and in spite of the danger of becoming fragmentary, our description will be mainly based on the electron micrographs published here. The basic data of this work is the result of several hundred electron micrographs taken from about 1400 explants. Topographically considered, and for the sake of description, our material may be classified as follows: a) within the cell body of the neurone; b) in the nerve fibers growing into the surrounding medium; c) at the level of the growing ends of the nerve fibers.

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[1] The culture technique of nervous tissue was started in this Department by Prof. Giuseppe Levi. We want to express here our gratitude and our admiration for the fundamental contributions he made to this field of research.

[2] This type of fixation produces little or no artefacts visible with the optical microscope and according to Porter *et al.* (31) is the best suited for electron microscopy of cells in tissue culture.

Observations on the ultrastructure of the nerve cell body

A. *General Description.* Figs. 1 and 2 show at low magnification two typical stages in the development of nerve cells *in vitro*. In both cases the nucleus and the region surrounding the nucleus is too thick and dense for the electron beam. Ultrastructural details can only be recognized in peripheral regions where the cytoplasm becomes expanded in thin membranes.

Fig. 1 shows a monopolar neuroblast of a 7 day old embryo having at the lower end the growing axon (*Ax*) typically enlarged at the extremity, and on the opposite region a membranous expansion of cytoplasm in which mitochondria (probably also round fat droplets) and a very thin fibrillar ultrastructure can be recognized. Fig. 2, from a 14 days old embryo shows a typical multipolar neuroblast which has migrated into the surrounding fibrin clot (see the fine fibrin fibrils in the background). The cytoplasm is in continuity with several long expansions in which it is impossible to differentiate the axon from the dendrites. Short finger-like expansions may be observed, some of which are beyond the resolution of the optical microscope. This cell is too thick to show fine details within the cytoplasm. All the other cells or cell regions which have been photographed show earlier stages of differentiation than those in Fig. 1 or stages that are intermediate between those indicated in Figs. 1 and 2.

The stages of differentiation of the different cells do not depend entirely on the age of the explant. Although in cultures of 3 to 4 days most of the cells are undifferentiated, in some cases the cell may show a definite fibrillar differentiation (Fig. 5). In explants from 6 to 8 days old embryos most of the cells show a definite differentiation of fibrous material (Fig. 7), but also many of them show early stages in fibrogenesis (Figs. 3, 4). Finally in embryos of 12 to 15 days (Fig. 6) the fibrillar material is generally very much developed within the cytoplasm.

The description will be made according to the degree of differentiation as reflected by the disposition and amount of fibrous material within the cytoplasm.

B. *Early stages in fibrogenesis.* According to most authors the deposition of the silver stained fibrous material starts at the juxtanuclear region. This makes the electron microscope observation difficult. Our description is thus mainly confined to the early process of fibrogenesis in membranous expansions of the cytoplasm. However, there is evidence that the observed development of the fibrous differentiation is probably similar near the nucleus.

In cells in which fibrogenesis is probably at one of the earliest stages, contrasting with the almost homogeneous matrix of the cytoplasm, there are regions of lower electron density in which very fine strands of dense material are present. Similar strands can be observed deeper in the cytoplasm, penetrating in the perinuclear region where mitochondria are also observed. In this thicker region resolution is poor. At high magnification it is possible to resolve the fine strands into short chains of a microvesicular material. This fact is illustrated in Figs. 3 and 4. In Fig. 3 the area of fibrogenesis (*f*) is more developed, and occupies the larger part of the cytoplasmic expansion. The material of higher electron density is constituted by isolated micro vesicles or short chains of them, showing a tendency to fuse into short elongated double-edged cylindrical figures. This material is accumulated also at the extremity of the cell expansion where finger-like processes appear. The region of fibrogenesis is generally devoid of mitochondria. However, in some cells one or more mitochondria may enter into the emitted processes accompanying the microvesicular and fibrous material.

Figs. 4 a and 4 b show at higher magnification the early stages in fibrogenesis. The high resolution obtained in this figure is probably due to the fact that it is a very thin membranous fragment of the cell, very likely adherent to the film and splitted on stripping it. The macromolecular material of high electron density appears on the background as short strands, mainly constituted by rounded or eliptical microvesicles (*mv*) with a denser edge and of about 300 to 500 Å in diameter.

TEXT TO FIGURES

Fig. 1. Electronmicrograph of a typical monopolar neuroblast. Toward the bottom of the figure the axon (*Ax*) with the enlarged growing end. On the upper side the cytoplasm expands into a membranous sheet in which mitochondria and a fine fibrous material are found. Culture No. 1037, rhomboencephale of a 7 days old embryo grown on parlodion in 1/6 plasma-Tyrode for 24 hours. Fixation with osmium vapor.

Fig. 2. Electronmicrograph of a multipolar neuroblast. Long branching processes and short submicroscopic processes of the cytoplasm are observed. On the background fine fibrin fibers. Culture No. 1013, medulla oblongata of a 14 days old embryo grown on parlodion in 1/6 plasma-Tyrode for 24 hours. Fixation in osmium vapors.

Fig. 3. Cytoplasmic expansion of a young neuroblast emitting fine amiboid processes. A large part of the matrix contains microvesicles and short chains of microvesicles of high electron density (*f*). The prefibrillar material tends to enter and is packed into two growing expansions of the cytoplasm. Mitochondria (*mi*) are found at the upper and lower end of the figure. Culture No. 892, mesencephale of a 7 days chick embryo grown on parlodion in 1/6 plasma-Tyrode. Fixation in osmium vapors.

Figs. 4 a and 4 b. Membranous fragment of the cytoplasm of a young neuroblast. In the matrix a dense fibrillar material appears which is disposed in microvesicles (*mv*), short chain of microvesicles and short segments (*sn*) of double-edged cylindrical structures (neurotubules). Culture No. 1035, rhomboencephale of a 7 days old chick embryo grown on parlodion in 1/6 plasma-Tyrode, fixation for 24 hours in osmium vapors. Fig. 4 a 17 000 × ; Fig. 4 b 30 000 ×.

Fig. 5. Membranous cytoplasm of a neuroblast in which a definite fibrillar material is present in the form of short segments of neurotubules (*sn*), isolated neurotubules of 450 Å in width (*n*) and bundles of neurotubules [submicroscopic neurofibril (*nf*)]. Culture No. 1032, rhomboencephale of a 4 days old chick embryo grown on parlodion in 1/6 plasma-Tyrode. Fixation in osmium, 11 000 ×.

Fig. 6. Membranous fragment of a developed neuroblast in which isolated neurotubules (*n*) and bundles of neurotubules [neurofibrils (*nf*)] of 0.11 μ to 1 μ in diameter are apparent. Culture No. 904 of an 12 days old chick embryo grown on parlodion in 1/6 plasma-Tyrode plus extract. Fixation in osmium vapors.

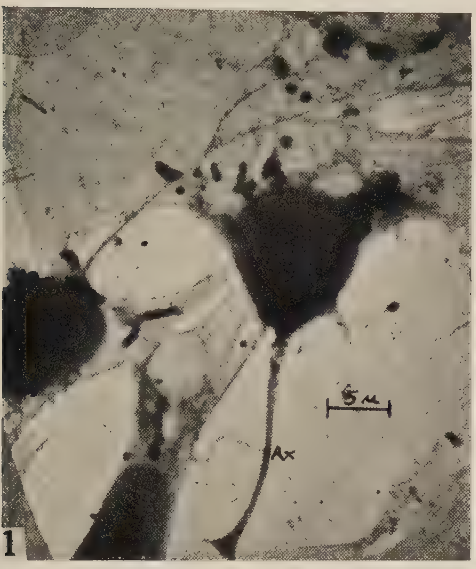
Fig. 7. Membranous fragment of a developed neuroblast. One neurofibril (*nf*) and isolated neurotubules (*n*) covering the largest area of the figure. Culture No. 1366, rhomboencephale of a 7 days old chick embryo grown on parlodion. Fixation in osmium vapors.

Fig. 8. Two nerve fibers showing parallel packed neurotubules. One of the fibers shows one small swelling in which the structure is maintained (lower part), and another in which this is totally destroyed (upper part). The upper right lines indicate cross banding of neurotubules. Culture No. 311, mesencephale of a 6 days chick embryo grown for 24 hours on formvar in 1/10 plasma-Tyrode. Fixation in osmium vapors.

Figs. 9 and 10. Several nerve fibers ranging in diameter between 100 and 300 mμ. Dense parallel lines indicate edges of neurotubules (*n*). Cultures No. 788, mesencephale of a 7 days old chick embryo grown for 24 hours on parlodion in 1/6 plasma-Tyrode. Vital staining with methylene blue. Fixation in osmium vapors. Fig. 9 at 26 000 × ; Fig. 10 at 19 000 ×. Inserts *a* and *b* are neurotubules obtained by sonic disintegration from rat tibial nerves fixed in formaldehyde.

Fig. 11. Growing end of a nerve fiber showing the enlarged terminal mass filled with microvesicles (*mvz*) and thin growing processes in which segments of neurotubules are found (*sn*). Culture No. 725, mesencephale of an 8 days old chick embryo grown for 18 hours on parlodion in 1/12 plasma-Tyrode. Fixation with osmium vapors.

Fig. 12. Growing end of a nerve fiber. Some of the terminal processes undergo degeneration into clear vesicles (*dv*), others are breaking into round dense masses (*dm*). Culture No. 727, similar to Fig. 11.



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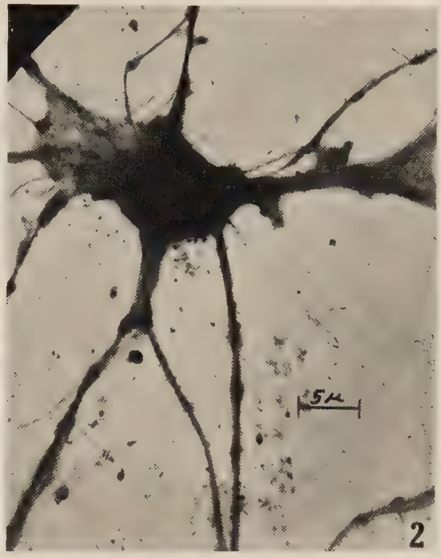

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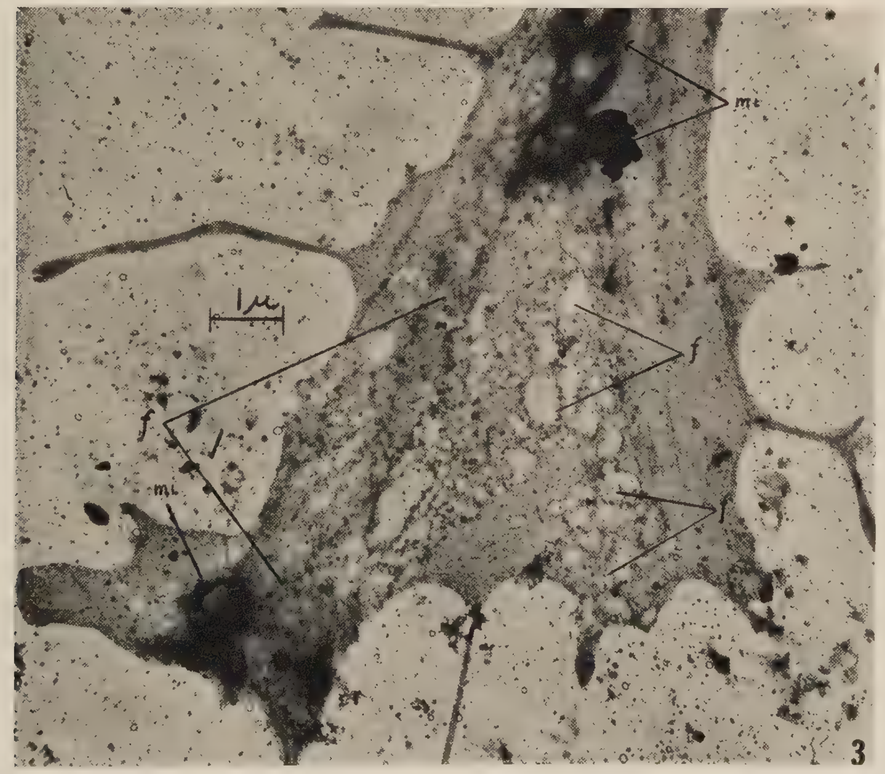
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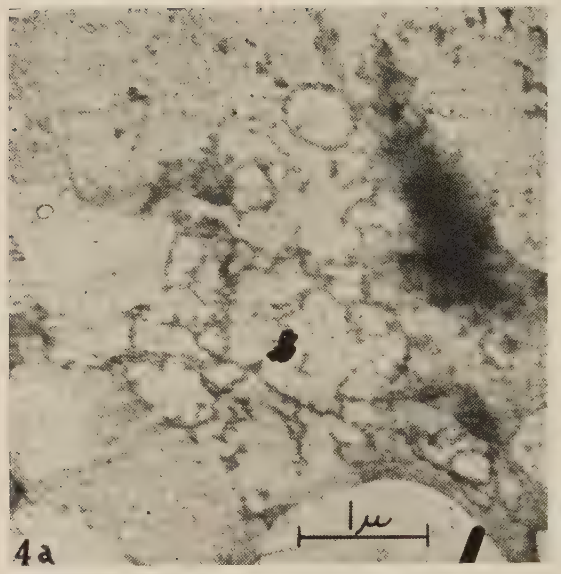
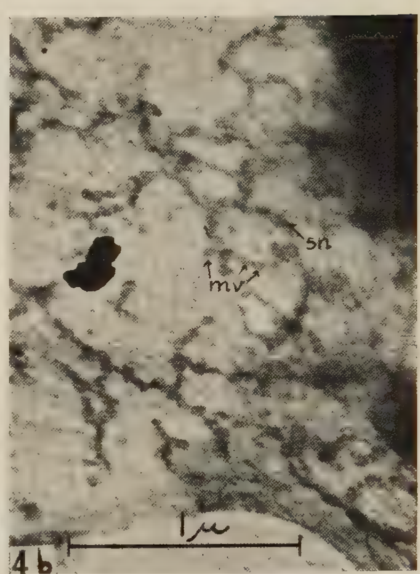
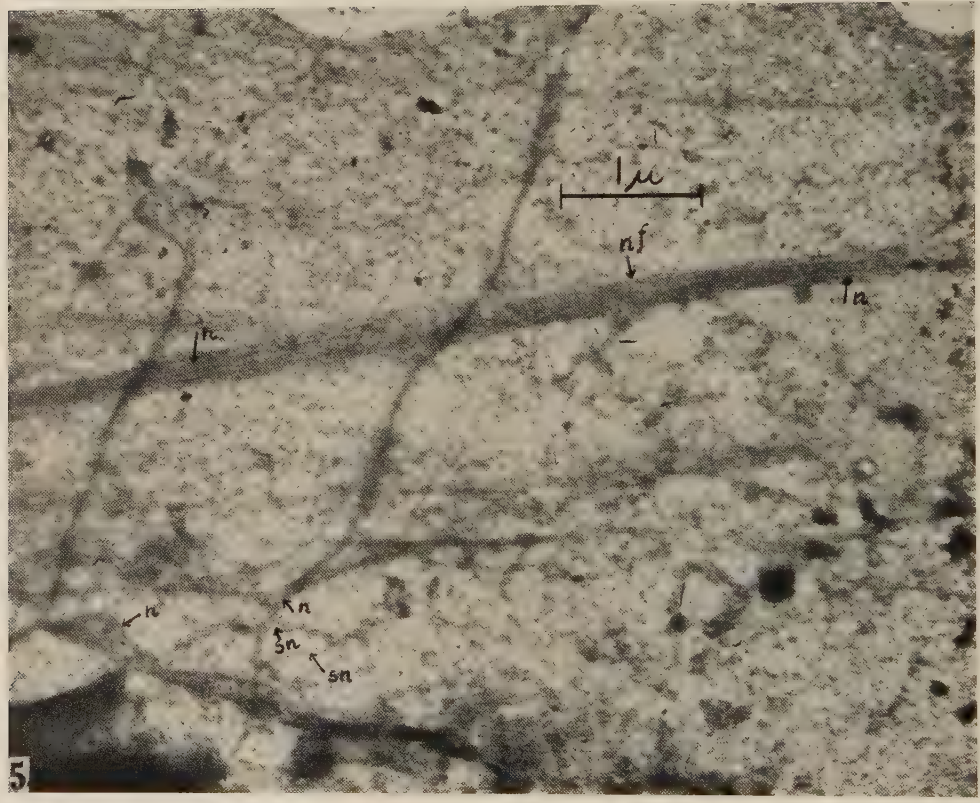
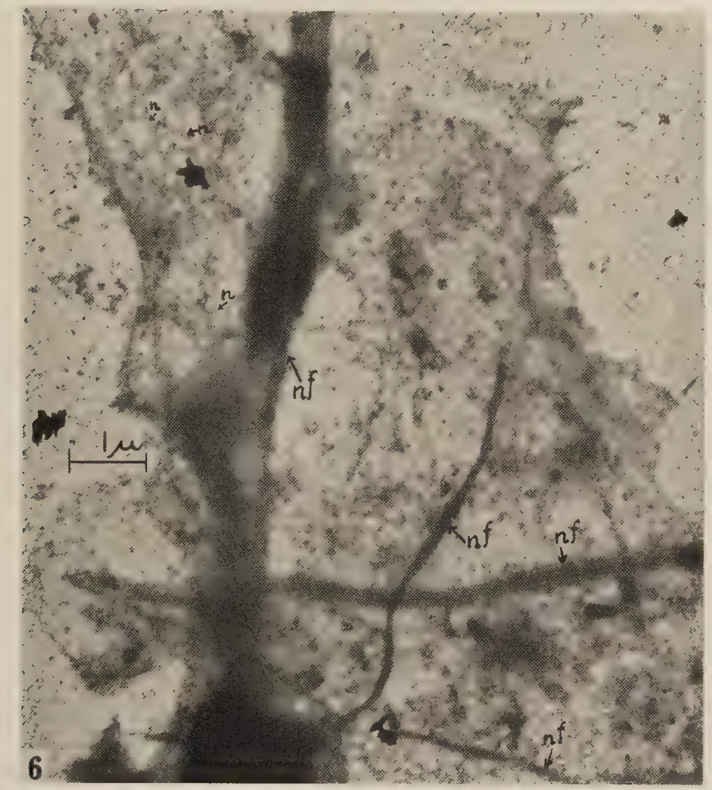
3

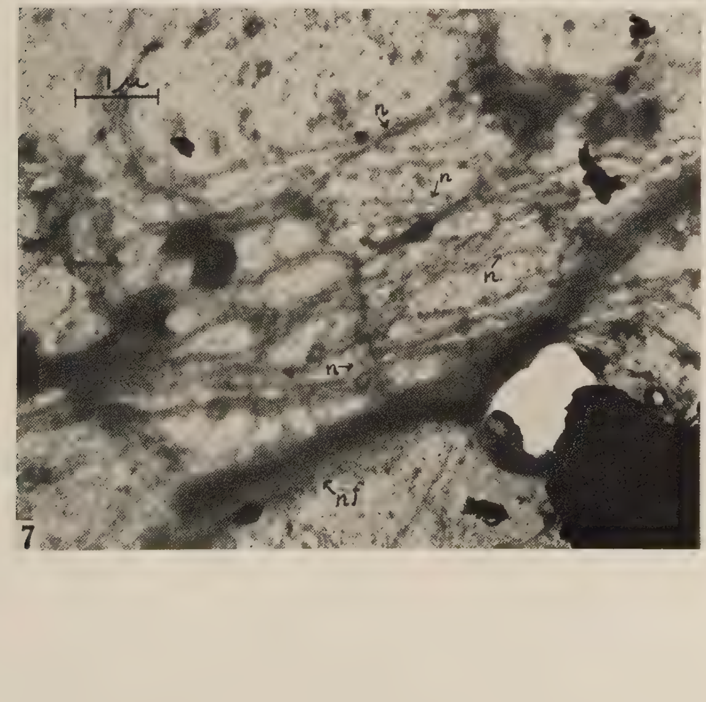

1μ
4a


sn
mv
1μ
4b


1μ
nf
n
n
n
n
sn
sn
5


n
n
n
nf
1μ
nf
nf
nf

6



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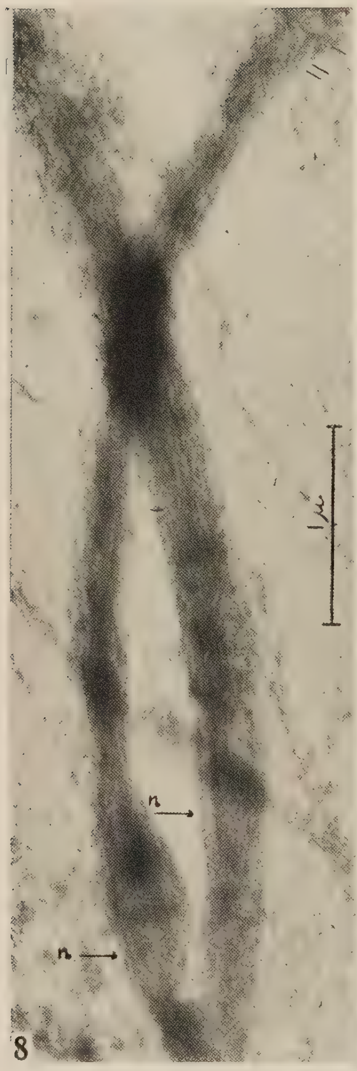

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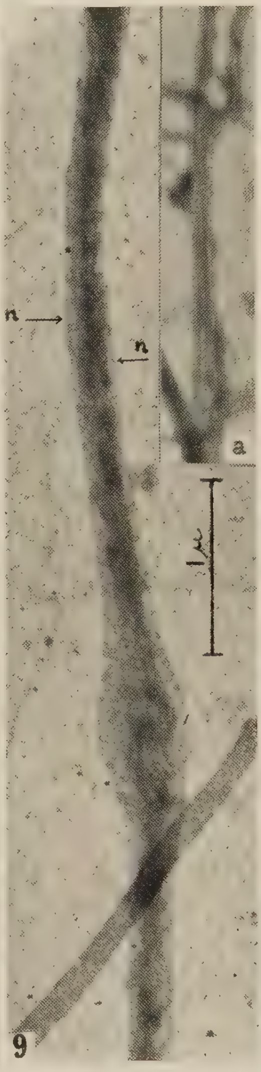

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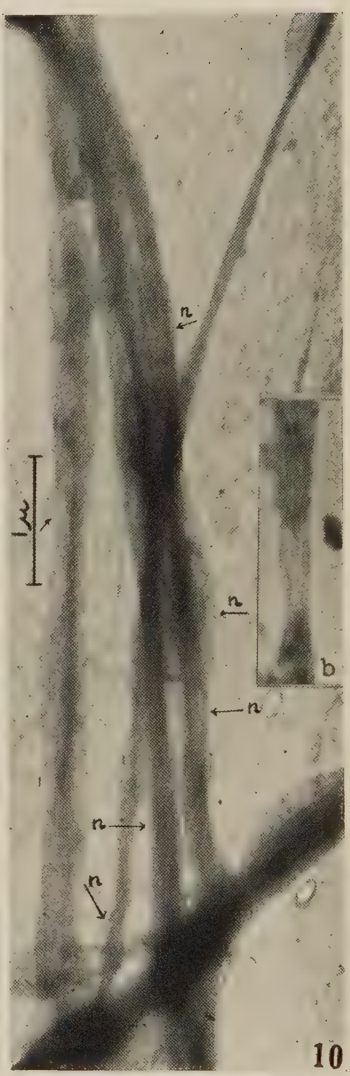

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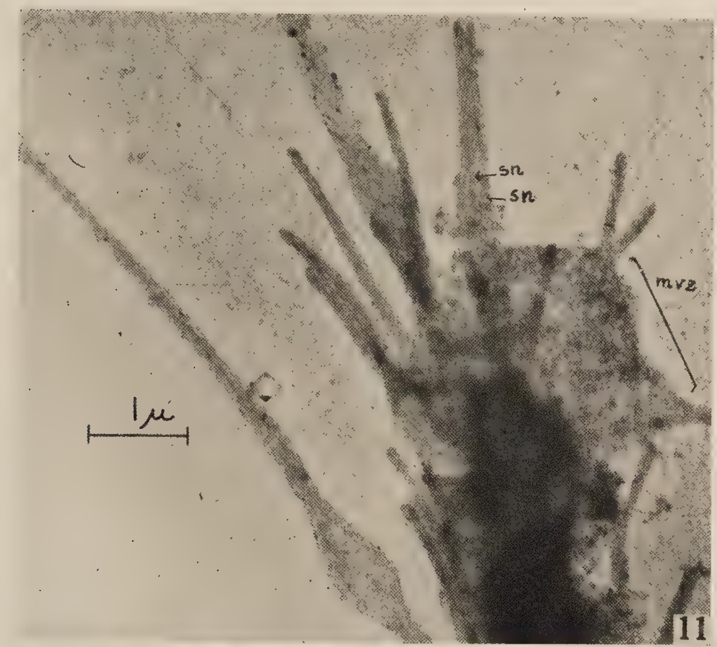
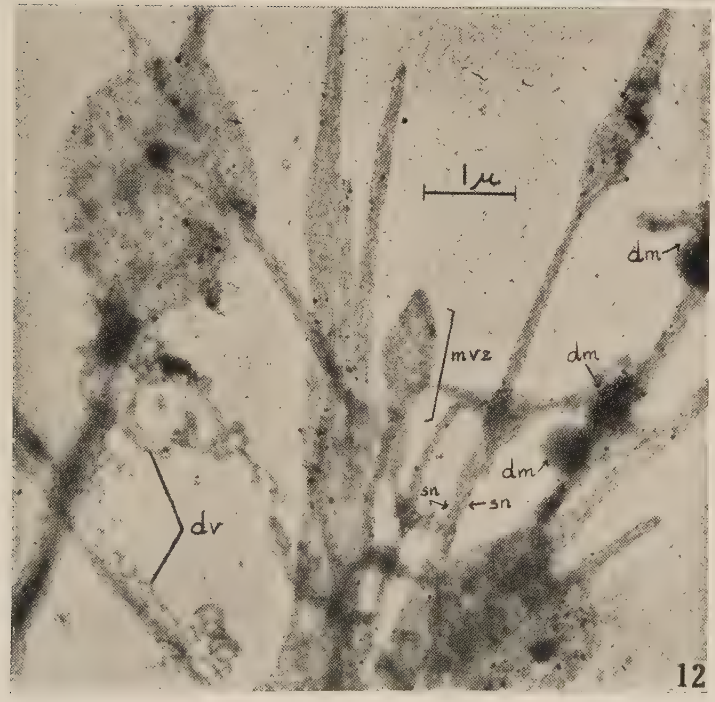


11


1μ
dm
mvz
dm
dm
sn
sn
dv

12

These microvesicles may be isolated, but generally they are attached into short chains with a tendency to fuse. Apparently as a result of the coalescence of these microvesicles, short double-edged cylinders can be formed that may be interpreted as segments of neurotubules (*sn*).

In more differentiated cells, in addition to chains of microvesicles and cylindrical segments of neurotubules (*sn*), definite fibrous elements appear. As can be seen in Fig. 5, these are long cylindrical structures of about 450 Å in diameter with dense edges and a core of lower electron density, similar to those described in fragmental nerves (see Figs. 10 a and 11 b), and in thin sections of nerve fibers (12, 9, 10). For this reason they will be called *neurotubules* (*n*). Neurotubules may be found isolated within the cytoplasm, but they have a tendency to form bundles which can be considered related to neurofibrils (*nf*). In Fig. 5 there is a bundle of neurotubules of 0.25 μ in diameter, just at the limit of the resolving power of the optical microscope.

C. *Later stages in fibrogenesis. Neurofibrils.* Observation of more advanced stages of differentiation is hampered by the increased thickness of the cell. We have electronmicrographs of more or less intact cells in which large amounts of dense fibrous material similar to the neurofibrils can be observed. Definition, however, is poor. Higher resolution is obtained in membranous fragments of cells resulting from the stripping technique used (Figs. 6, 7). All our evidence indicates that the fibrous differentiation in cells is constituted by neurotubules which can be found free in the cytoplasm or condensed in bundles of varying diameter. Some of these correspond to the size of the neurofibrils of classical Histology. In Fig. 6, in addition to a number of isolated neurotubules (*n*) of about 400 Å, bundles (*nf*) of about 1 μ, 0.35 μ, 0.15 μ, and 0.11 μ can be observed. Thus it seems logical to think that the larger bundles would be resolved as neurofibrils at the level of the optical microscope, while the smaller ones probably are entirely submicroscopic.

In Fig. 7 only one condensed bundle of neurotubules (*nf*) is observed, while most areas show isolated neurotubules (*n*).

Observations on the ultrastructure of nerve fibers

A short description of the ultrastructure of nerve fibers grown *in vitro* has been published previously (11). Nerve fibres growing in contact with the film are considerably flattened and thus more or less permeable to the electron beam. This ribbon-like aspect to the fibers can be particularly appreciated vith the shadow casting technique.

Nerve fibers varying in diameter between a few microns down to 0.1 μ, have been observed. It is an interesting fact that we also found collaterals and growing tips of a diameter beyond the limit of resolution of the optical microscope.

Fig. 8 shows two nerve fibers of approximately 0.3 μ in diameter. The dark parallel lines within the fibers limit double-edged cylindrical structures (*n*), identical with neurotubules found in cultured nerve cells and in the peripheral nerve fibers of vertebrates. The diameter of the neurotubules varies between 300 and 500 Å. Cross striation of neurotubules is barely visible in the upper part of the figure.

Figs. 9 and 10 show fibers stained with methylene blue ranging from 0.3 o 0.1 μ in diameter. These seem to be composed of a few parallel neurotubules (*n*). Small inserts *a* and *b* in figures 9 and 10 show neurotubules from rat tibial nerves obtained by fragmentation with sonic oscillations.

Another component frequently found in nerve fibers are mitochondria. Describing the nerve cells, we pointed out that frequently mitochondria can be seen to enter into processes of the cytoplasm together with the fibrous material. They can also be observed along the entire length of the fiber up o the growing end. They generally appear as rods on one side of the fiber, sometimes bulging on the surface. They can be easily distinguished by a very high electron density and sharp limits.

The diameter of the nerve fiber is not uniform. Very frequently swollen eliptical regions can be observed, similar to those described with the optical microscope. Some of them appear to be caused by the action of different chemical or physical agents, while others represent stages in the degeneration and disintegration of the nerve fiber. With the electron microscope one can observe different aspects and degrees of swelling. For example in Figs. 8 and 9 there is only a moderate swelling and the internal structure does not seem to be affected. In some cases the swelling puts in evidence the presence of a very thin membrane at the periphery of the fiber which is not observed normally. Regions of very marked swelling can be seen in other electronmicrographs. This increased swelling results in the destruction of the normal structure of the fibers, and appears as blisters which flatten down into a thin membrane. This is demonstrated by their low electron density and by the shadow-casting method.

In the regions in which collaterals are emitted, one generally finds an enlarged region of the fiber but with maintenance of the normal structure or the presence of a microvesicular material. Vital staining of the cultures with methylene blue results in an increase in the electron density of the fibers and their components (Figs. 9, 10).

A few observations of older cultures show stages in the degeneration of the nerve fibers, frequently starting with the formation of blisters. Disintegration of the neurotubules gives rise to round clear degenerating vesicles (*dv*) of 500 to 1 000 Å. This diameter is definitely larger than that found in the microvesicles which appear during fibrogenesis.

Ultrastructure of the growing end of the nerve fiber

The typical ending of nerve fibers growing *in vitro* is generally characterized by an enlarged terminal mass and by finger-like processes with a fan shape disposition (Figs. 11 and 12). The enlarged mass appears to be mainly constituted by a tightly packed microvesicular material (*mvz*) similar to that observed in the nerve cell in the early stages of fibrogenesis. This material is in continuity with the processes where it becomes organized into segments of neurotubules (*sn*) of varying length. Frequently, the largest processes have enlarged regions near the base (Fig. 11), along the length or at the end (Fig. 12 *mvz*). These regions also contain microvesicular material. Sometimes, at the level of the terminal mass, and in addition to microvesicles, an amorphous material of high electron density and mitochondria may be present.

The growing processes are of varying length and diameter. In fig. 11 there are processes of less than one micron to more than 10 μ in length. The diameter of most of them varies between 0.1 μ and 0.14 μ being entirely submicroscopic. The two larger ones in Fig. 11 are of about 0.27 μ and 0.35 μ at the level of the base and could be resolved with the optical microscope. In the first one the tip is submicroscopic (0.17 μ).

Within the growing process short segments of neurotubules (*sn*), and in some cases typical neurotubules of 300 to 500 Å in diameter can be observed. Frequently neurotubules are not straight but follow a curved or wavy path within the process. In some instances the growing processes undergo changes that can be interpreted as of degenerative nature. As seen in Fig. 12 *dv* the process may become disintegrated into clear vesicles in a way similar to the degeneration of nerve fibers and neurotubules.

In other cases, the end of the process enlarges into a very dense spheric mass (Fig. 12 *dm*), which apparently may become isolated by rupture of the connection. In fact, similar dense homogeneous masses can be observed near the nerve endings (Fig. 12 *dm*), having no continuity with the processes. In some electron micrographs several of these spheres are isolated but disposed in one line probably occupying the place of the process from which they originated.

DISCUSSION

Fibrogenesis of the specific substance of the neurone: A detailed analysis of the literature on the embryonic development of nerve cells is beyond the scope of this paper (4, 30). It is well established that in the chick embryo the silver stained neurofibrillar material appears in some indifferentiated cells at the 3rd day of incubation and even earlier (3, 5). In explanting nervous tissue from embryos of 3 or more days, one obtains a complex cell population in which there are indifferentiated nerve cells and neuroblasts with different degrees of differentiation (20). This complexity refers not only to the cells but also to the nerve fibers.

According to Levi (23), in explants from the second to the sixth day, differentiation continues *in vitro* giving rise to new neuroblasts and nerve fibers. In later stages nerve fibers are mainly regenerated. Our observations with the electron microscope are in accordance with the previous facts.

From the ultrastructural point of view, our results seem to indicate that fibrogenesis starts with the appearance of fine strands of a material of high electron density within the more or less homogeneous matrix. This material consists of rounded and eliptical microvesicles of 300 to 500 Å in diameter which are isolated or, more frequently, attached into short chains. Apparently the coalescence and the probable fusion of this string of microvesicles produces double-edged cylindrical structures that can be interpreted as segments of neurotubules. The microvesicular material is at first localized in definite areas of the ground cytoplasm, but later may occupy large regions of it. This fact, in addition to differences in size, electron density and disposition, distinguishes this material from the endoplasmic strands of granules and vesicles observed by Porter *et al.* (31) in fibroblast-like cells.

Two main mechanisms of macromolecular aggregation seem to lead to the formation of fibrillar material. One is the aggregation, laterally and longitudinally, of smaller asymmetrical units (filaments, etc.). The other results of the linking of globular macromolecules in a definite stereochemical pattern. Recent work on the extracellular synthesis of collagen type fibrils (33) and in the fibrinogen-fibrin transformation (32) show that this last type of mechanism is probably the most frequent in the first stages of fibrogenesis.

Our results tend to indicate that the *intracellular synthesis of fibrillar ma-*

terial in neuroblasts may also be the result of a similar mechanism leading from the aggregation of microvesicles into the formation of fibrous neurotubules.

At the beginning of the century a large number of studies were dedicated to establish a relationship between the origin of the neurofibrillar substance and different cell constituents. Particularly pertinent was the theory of the mitochondrial origin of neurofibrils, as proposed by Meves and supported by several authors (Lit. in Cowdry (5)). On cytological evidences Cowdry (5) denied this theory and reached the conclusion that in all probability neurofibrils develop in the ground substance. Our results conclusively demonstrate that the fibrillar material of the nerve cell originates within the matrix of the cytoplasm and has no direct relationship with mitochondria or other formed bodies.

This conclusion puts the problem of fibrogenesis on cytochemical and biochemical bases. Using microspectrographic analysis, Hydén (19) demonstrated that in the early stages of development of the unipolar and multipolar neuroblasts, comparatively small amounts of nucleotides are present in the cytoplasm and that there is only a slight increase in the total amount of protein. Later on, coinciding with a large increase in size of the cell and with the development of the nucleolus, there is a marked increase in ribose nucleotides and proteins in the cytoplasm.

Most of the young neuroblasts studied by us probably belong to the first group as described by Hydén. Although fibrogenesis has already started, there are not marked changes in the nucleotide content of the cell. However, and because of the difference in material and conditions, no definite correlation can be established between these results and our findings.

Thus it would seem of great interest to make a coordinated effort, by using quantitative cytochemical techniques and ultrastructural analysis, on the problem of nerve fibrogenesis.

Morphological significance of neurotubules and neurofibrils: A review of the literature regarding the morphological significance of the neurofibrils will not be considered here (30, 24). It seems beyond doubt that neurofibrils can be observed *in vivo* in special materials and conditions (2, 6, 20, 24, 37) and that they are not artefacts due to the technical procedures employed, although these procedures may introduce certain changes. Microsurgical methods have demonstrated that neurofibrils are gelled, mechanically resistant structures and that they can be displaced with the needle of the micromanipulator (6, 25). These physical properties certainly depend on the submicroscopic organization of neurofibrils as shown with the electron microscope.

Our observations on neuroblasts tend to show that the neurotubules can be found isolated in the cytoplasm, but more frequently they become laterally associated into bundles of varying sizes, the larger ones having dimensions similar to the neurofibrils. These facts seem to point to the conclusion that the *neurotubules is the ultrastructural unit of the specific substance of the neurone, while the classical neurofibril results from the association in a bundle of a number of neurotubules large enough to be resolved with the optical microscope.*

Fibrogenesis and growth of the nerve fiber: In a recent experimental work on adult regenerating nerve fibers, Weiss and Hiscoe (36) reached the conclusion that the growth of the axoplasm (protoplasm of the nerve axon) occurs solely at the base of the fiber, in the nucleated part of the cell body. This is certainly not the only mechanism of growth in embryonic and particularly in cultured nerve fibers. In this case the growth of the fiber depends also on the autonomic activity of the growing end which has a pronounced amiboidism. Levi (17) has shown that if a growing fiber is sectioned with micromanipulation the distal isolated end may continue its amiboid activity for several hours and may even increase in size.

Our findings of a microvesicular material, similar to that found in the cytoplasm of neuroblasts in fibrogenetic activity, indicates that *probably at the terminal growing end a similar process of biological synthesis occurs which gives rise to fibrous neurotubules.* Other centers of fibrogenesis are probably present in the regions where collaterals are emitted. This coincides with an increase in the diameter of the nerve fiber.

Our observations show that from this expanded, and probably more fluid end, finger-like pseudopods are emitted in which segments of neurotubules can be observed. The pseudopods are at first entirely submicroscopic and only by an increase in their diameter they may become partially visible with the optical microscope. This fact was suspected by Mossa (28) who with dark field microscopy could observe that the terminal filaments are longer and finer than when observed with direct light.

Degeneration of cultured nerve fibers: Along the fibers, enlarged regions are present which have different structure and probably different significance. We have already suggested that the enlargements found at the growing end and at the level of the collaterals are probably related to the formative properties of these regions.

Fischer (17), mentions the fact that varicosities along the fiber may be formed and then disappear and that sometimes this process assumes the character of a peristaltic movement in the distal direction. Martinovic (26) finds that swellings may be caused by different agents such as alcohol, ether, formalin, and that when present *in vivo* they indicate a process of degeneration.

Our observations show that at the level of the swelling the ultrastructure may be sometimes quite normal. However, when this process is very intense, one may observe the complete disintegration of neurotubules within the blister. Observation of nerve fibers which are undergoing a process of degeneration and disintegration shows that neurotubules have a tendency to break into clear round vesicles, as shown by De Robertis and Schmitt (12) in Remak fibers from human sympathetic nerves degenerating "*in vitro*."

CONCLUSIONS

Nervous tissue from 3 to 14 days old chick embryos was cultured *in vitro* and studied under the electron microscope. The ultrastructure of the cell body, the nerve fiber, and the growing terminal end was studied.

I. Cells in different stages of differentiation, as indicated by their expansions (monopolar, multipolar neuroblasts) and by the disposition and amount of fibrillar material have been photographed.

Early fibrogenesis is characterized by the appearance in the homogeneous matrix of the cytoplasm of fine submicroscopic strands of high electron density. This dense material is constituted by isolated microvesicles of 300 to 500 Å in diameter, or by short chains of them showing a tendency to fuse into double-edged cylindrical structures. In more differentiated cells, definite long cylindrical fibrous elements also appear. These elements have dense edges and a diameter from 300 to 500 Å. Because of their similarity with previously described structures in fragmented nerves (12) and in thin sections of the axon (9, 10) they are called *neurotubules*.

Neurotubules may be isolated in the cytoplasm but more frequently they show a tendency to form bundles of 0.1 μ to 1 μ in diameter. The thicker bundles can be considered as typical neurofibrils, while the smaller ones are entirely submicroscopic. The morphological significance of the neurofibrils is discussed.

The probable mechanism of fibrogenesis is discussed on the basis of the literature dealing with the formation of extracellular fibrils by the aggregation of globular macromolecules.

It is demonstrated that the fibrillar material of the nerve cell originates within the ground substance of the cytoplasm and has no direct relationship with mitochondria or other formed bodies.

II. The electron microscope analysis of nerve fibers growing in tissue culture demonstrates that a number of fibers and most of the collaterals and the growing tips are of submicroscopic dimensions. The nerve fiber appears to be constituted by parallel neurotubules closely packed. Mitochondria and a peripheral plasma membrane may also be observed.

Along the fibers enlarged regions may appear. These regions may or may not maintain their ultrastructure according to their degree of swelling. Methylene blue staining increases the electron density of the nerve fibers and their components.

Vesicles of 500 to 1 000 Å in diameter have been observed in different stages of degeneration and disintegration of the nerve fibers.

III. The terminal growing end usually shows an enlarged mass and finger-like processes. The enlarged mass contains a tightly packed microvesicular material similar to that observed within the cell in early fibrogenesis. An amorphous dense material and mitochondria may also be present.

Most of the growing processes are of submicroscopic dimensions and show short segments of neurotubules, and in some cases typical neurotubules of 300 to 500 Å in diameter. Degeneration of these processes is frequently observed.

These observations seem to support other data from the literature indicating that the terminal growing end is a center of fibrogenetic activity.

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THE ULTRA-VIOLET ABSORPTION OF LIVING CHICK FIBROBLASTS DURING MITOSIS

H. G. DAVIES

University of London, King's College, London

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Recently Jacobson and Webb (7, 8) have investigated the distribution of the two types of nucleic acid in cells during mitosis; the materials used included chick fibroblasts grown in tissue culture and the methods were those of fixation and staining. This work caused the author to make a further study of mitosis, see also (15), in living chick fibroblasts *in vitro* by ultra-violet microscopy; the results have confirmed some of the observations described by Jacobson and Webb.

The early studies on living cells by ultra-violet microscopy, for example those of Wyckoff *et al.* (15, 16) and Lucas and Stark (9), were made photographically in a purely qualitative way. Caspersson (1) placed the entire procedure of microspectrography on a quantitative basis; in this way a study of living blood cells was made by Thorell (11). The recent observations of Ludford and Smiles (10) on living tumour cells (not in tissue culture) in which attention was drawn to the many cytoplasmic inclusion bodies, were illustrated by a series of beautiful photomicrographs. When ultra-violet microscopy is applied to living cells under conditions in which the damage from radiation is negligible, a picture is obtained of the distribution of ultra-violet absorbing constituents undistorted by any redistribution or loss that may occur on fixation and with subsequent procedures. However, as is well known, the method is subject to certain limitations. In the first place it lacks specificity, for example it does not distinguish between mononucleotides and the two types of nucleic acid; in the second place geometrical difficulties arise in that, while the fine structural details in any photomicrograph are due to those elements lying in the plane on which the microscope is focussed, general density gradations are also determined by out of focus regions of absorption above and below this plane, and the magnitude of these may be difficult to ascertain. Commoner (3) has considered the special case where the living cell has a well defined and measurable geometry. When the ultra-violet microscope technique is applied to fixed cells these difficulties may be overcome to some extent by the use of specific enzymes and by sectioning away the interfering regions of absorption. The results obtained in the present investigation are therefore complementary to those obtained by other techniques.

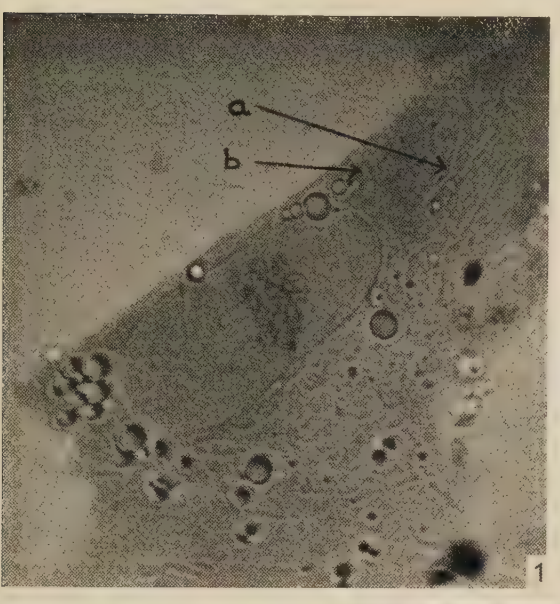
MATERIAL AND METHODS

Fragments of heart from 11–12 day chick embryos were grown by a modification of the hanging drop method devised by H. B. Fell for cytological studies. Equal parts of fowl plasma and chick embryo extract were mixed in a centrifuge tube and allowed to clot in the incubator for 30 minutes. The clot was then broken up and after a further 15 minutes incubation the fluid which exuded was withdrawn. A mixture of plasma and embryo extract was then deposited in the form of a horseshoe on a 0.1 mm coverslip. When the mixture had clotted the space within the horseshoe was filled by a thin layer of the exudate in which the explant was placed. The quartz slip was attached by a drop of Tyrode to a glass cover slip as in Maximow's technique, sealed to a hollow ground slide, and incubated with the coverslips downwards. After 24–48 hours when the explant was surrounded by a single layer of cells the quartz cover slip was sealed to a special culture chamber (6).

The ultra-violet microscope (12) used in this investigation was designed particularly for work on living cells. It had the following special features. The cells could be continuously observed with visible phase contrast illumination, growing and dividing in the culture chamber on the warm stage of the ultra-violet microscope. By means of auxiliary optical equipment and without change of objective, an ultra-violet photograph at any chosen wavelength between 240 mμ and 313 mμ could be rapidly taken in about one second after accurate focussing in visible phase contrast. Exposure times were of the order of half a second. In this way photographs were taken at 265 and 313 mμ of over sixty selected stages of mitosis and without any previous ultra-violet radiation to the cells. The photographs shown in the plates were taken under the following conditions. Fig. 1–13, excluding Fig. 6, were taken with the quartz, lithium-fluoride achromat (designed by Mr. Bracey of B.S.I.R.A.). N.A. 1.24, condenser N.A. 0.6, λ 265 mμ; and enlarged ×1 500 (Figs. 1–5) or ×1 700 (Figs. 7–13) from a negative at ×114 on Kodak Microfile Panchromatic 35 mm film; they were developed in D.19 b for 4 minutes at 20° C. Fig. 6 was taken with a reflecting monocentric objective, N.A. 0.5 condenser, N.A. 0.5, λ 265 mμ, and enlarged to ×1 100.

The densitometer traces in Text-fig. 1, 2, a, b were obtained with the recording densitometer constructed by Mr. P. M. B. Walker. By employing the linear portion of the film characteristic the transformation of photographic density to cell optical density is readily made, the measured film gamma being used.

Fig. 1. Intermitotic chick fibroblast showing phase effects on mitochondria.
Fig. 2. Intermitotic cell showing centrosphere and grape-like nucleolus. Optical density (O.D.) nucleolar area ≈ 0.25, O.D. clear cytoplasm ≈ 0.12, O.D. Nuclear area ≈ 0.11, O.D. centrosphere ≈ 0.07.
Fig. 3. Prophase: nucleolus and nuclear membrane still apparent.
Fig. 4. Late prophase: note the nucleolus and nuclear membrane have disappeared.
Fig. 5. Metaphase showing the relatively clear spindle area.
Fig. 6. Mouse fibroblast at metaphase showing the relatively clear spindle area.



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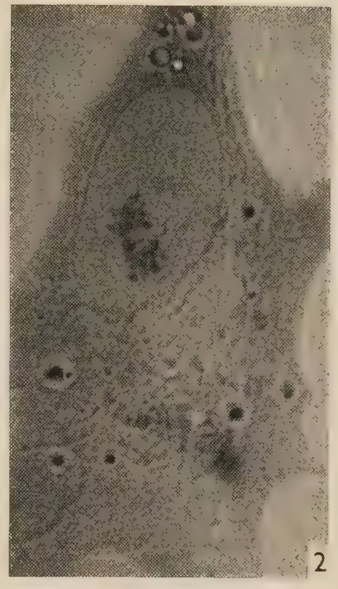

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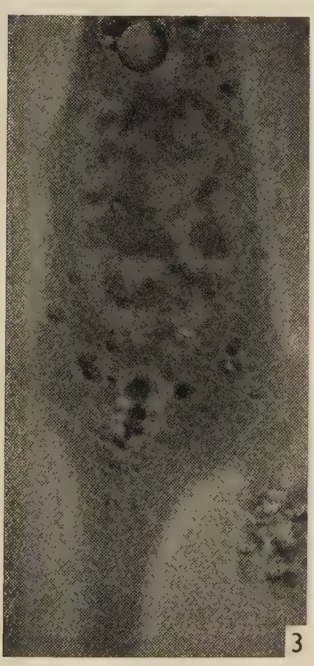

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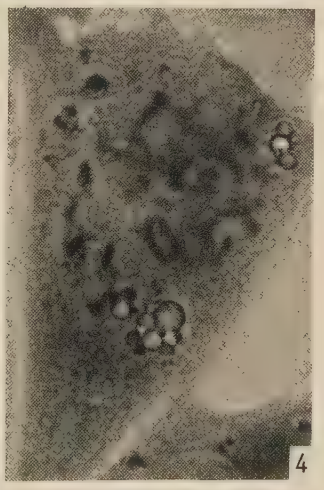

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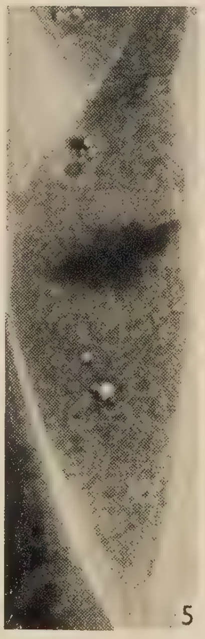

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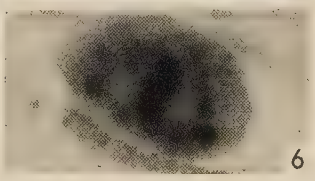

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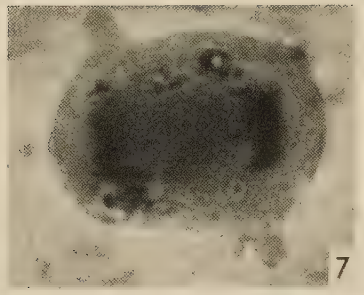
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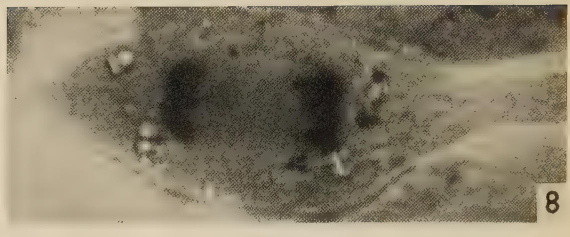
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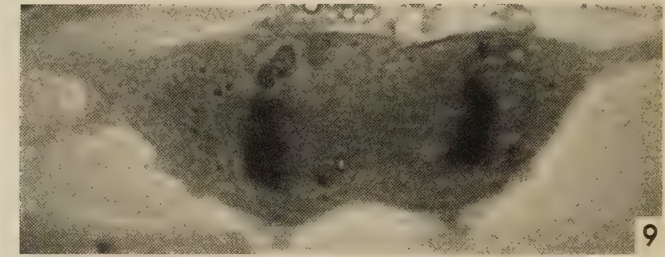
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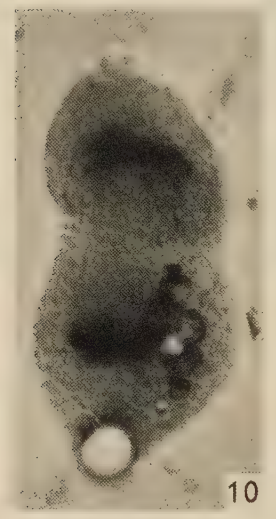
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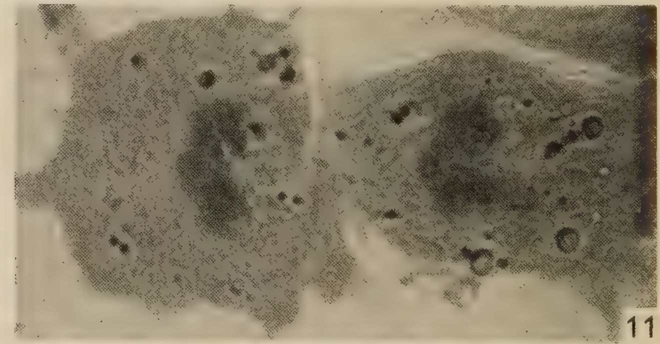
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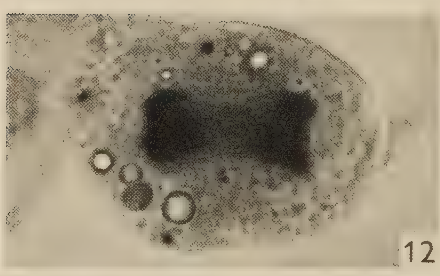
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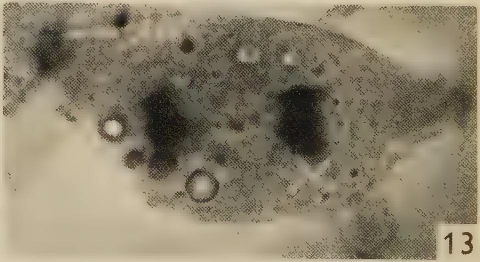
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RESULTS

Stages in the mitotic cycle of chick fibroblasts photographed at a wavelength of 265 mμ are shown in Figs. 1–11. The nucleus of the intermitotic cell (Figs. 1, 2) by comparison with that of mouse or newt fibroblasts, appears relatively homogeneous. One or two nucleoli and sometimes other structures which lie near the limit of resolution can be seen. The structure of the nucleolus varies considerably from a fairly uniformly absorbing body to one resembling a bunch of grapes. In the cytoplasm which absorbs homogeneously the centrosphere can often be distinguished as a less absorbing region adjacent to, and of the same order of size as the nucleus. Usually this area is the site of an aggregation of thin filamentous mitochondria, which together with fat droplets are the most conspicuous objects in the cytoplasm.

The appearance of the images of the mitochondria changes with focus, owing to phase contrast effects (13). This is demonstrated in Fig. 1; at *a* the mitochondrium appears as a non-absorbing filament with a dark halo, and at *b* as an absorbing filament with a light halo. At a slightly different focus the effect is reversed. Further work is required to find the extent to which these mitochondria, as they exist in the living cell, contain u.v. absorbing material; this may be difficult to determine since they are small bodies ($\approx 0.5\ \mu$ diameter) embedded in a medium which itself absorbs. The biochemical extraction data obtained from mouse liver cells (4) indicate the order of magnitude of the absorption in that particular material; considerable variation may be expected depending on the metabolic state of the cell. A mitochondrium of say 0.5 μ diameter, containing very approximately 3.5 per cent by dry weight of ribose nucleic acid and say 60 per cent water would have an optical density of only 0.015, which is near the limits of accurate detection.

At prophase (Fig. 3) the chromosomes appear as convoluted threads more absorbing than the nuclear background. The nucleolus and nuclear mem-

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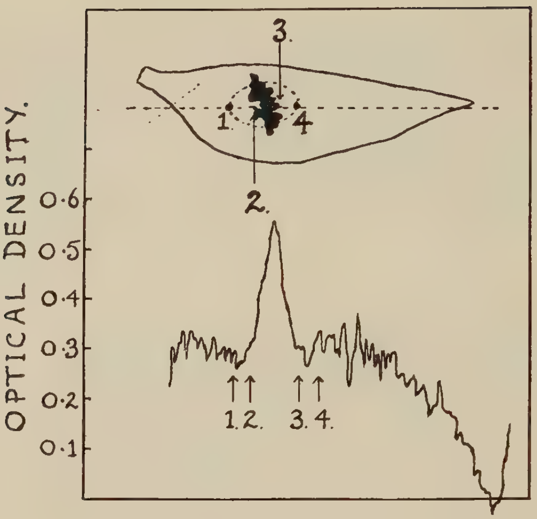
Fig. 7. Anaphase showing the area of greater absorption between the chromosome groups.
Fig. 8. Another anaphase cell similar to that in Fig. 7. Note the fairly sharp line of demarcation between the absorbing area and the adjacent cytoplasm.
Fig. 9. Cell at early telophase.
Fig. 10. Later telophase.
Fig. 11. Early reconstruction; the area of greater absorption has now disappeared.
Fig. 12. Anaphase showing area of greater absorption between the chromosome groups.
Fig. 13. Same cell as in Fig. 12 photographed 10 minutes after irradiation, with ultra-violet light λ 265 mμ. Note that the absorbing area seen in Fig. 12 has disappeared as a result of irradiation.



Text-fig. 1. Microphotometer record across the cell in metaphase shown in Fig. 5; line of traverse indicated by the dotted line in diagram. The spindle regions between 1, 2 and 3, 4 are seen to absorb relatively less than the adjoining cytoplasm.

brane which are to be seen in Fig. 3 later disappear (Fig. 4). The absorption of the chromosomes now increases and reaches a maximum at metaphase (Fig. 5) where they are to be seen in a more rounded cell lying in a plane at right angles to the coverslip. The spindle is without apparent structure and contrasts with the cytoplasm which is densely packed with the inclusion bodies. Elongated mitochondria are still to be seen.

Microphotometer records were made across six cells in metaphase. A typical trace obtained from the cell shown in Fig. 5 is reproduced in Text-fig. 1; this demonstrates that in the spindle region the optical density, which is proportional to the amount of absorbing material per unit area, is slightly less than in the adjacent cytoplasm. The conversion of this measurement to an exact estimation of the concentration of u.v. absorbing material in the spindle could only be made from precise geometric measurements of the shape of the cell and would include the amount of absorbing material above and below the spindle region; these factors could not be ascertained. However, we know that the metaphase cell is roughly ellipsoidal so that if the absorbing material were evenly distributed the absorption of the spindle area would be expected to be the same as or slightly greater than that of the adjacent cytoplasm. Therefore the total concentration of u.v. absorbing material in the spindle is certainly not greater than, and is probably less

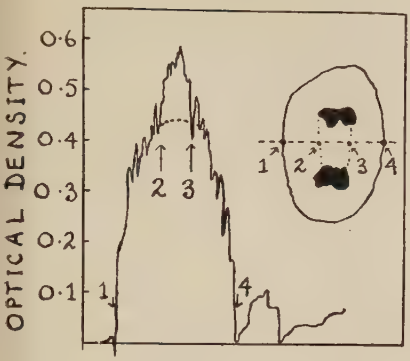


Fig. 2 a.

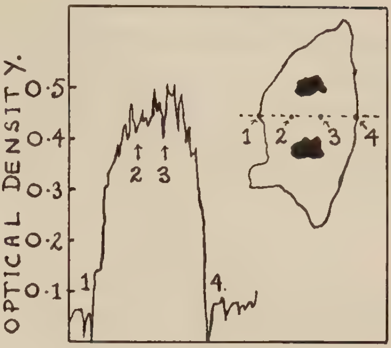


Fig. 2 b.

Text-fig. 2 a. Microphotometer record across the anaphase cell shown in Fig. 12; line of traverse is indicated by line 1–4 in the diagram. The trace shows between 2, 3 and increase over the absorption to be expected (dotted) from a homogeneous distribution of absorbing material in an ellipsoidal body.

Text-fig. 2 b. Microphotometer record across the same region of the same cell after irradiation (cf. Fig. 13). The trace shows that the higher concentration of absorbing material between the groups of anaphase chromosomes has disappeared.

than in the adjoining cytoplasm. Wilkins and Martin had noted in mouse fibroblasts that the spindle area was relatively non-absorbing (Fig. 6).

During anaphase (Figs. 7, 8, 12) the spindle region between the two chromosome groups is seen to be more heavily absorbing (i.e. has a greater density per unit area) than the adjacent cytoplasm. This region has been clearly observed in fourteen out of eighteen cells photographed; it is often sharply demarcated from the adjoining cytoplasm. Elongated mitochondria are seen throughout the cytoplasm. A typical microphotometer trace taken across the cell shown in Fig. 12 is reproduced in Text-fig. 2 a (full line); the dotted trace between points 2 and 3 indicates roughly the trace to be expected from an ellipsoidal body of uniform total concentration. It is concluded from the recorded trace that the region between the chromosomes contains a greater concentration of u.v. absorbing material than the adjoining cytoplasm. Other possibilities can be excluded, for example it is unlikely that the cell has bulges containing absorbing material above and, or below this region. Density changes due to structural arrangement of absorbing material as envisaged by Commoner (2) can be ignored at the comparatively low densities in the spindle region.

With the advent of telophase there is a simultaneous constriction of the absorbing region between the chromosomes and of the surrounding cytoplasm (Fig. 9). As telophase proceeds (Fig. 10) the interchromosomal absorbing material disperses and at an early stage of reconstruction (Fig. 11) no localised regions of higher concentration can be seen in the two daughter cells. The cells have now spread out and the chromosomes appear as fine threads occupying a larger area than at anaphase.

Changes are produced in the anaphase cell by prolonged exposure to ultra-violet light. A cell at anaphase (Fig. 12) was exposed to a dose of about 4×10^5 ergs. $cm.^{-2}$ (14 photographs): 10 minutes later (Fig. 13) the extra absorption between the chromosome groups had disappeared. On microphotometry the trace after irradiation (Text-fig. 2 b) resembled more closely the dotted theoretical curve (Text-fig. 2 a) roughly drawn for an even distribution throughout the cell volume: the continuous trace in Text-fig. 2 a is the record that was actually obtained from the cell in Fig. 12 before irradiation.

Studies at 313 mμ outside the regions of specific absorption show that the above conclusions are not affected by light scattering or refraction.

DISCUSSION

Wyckoff *et al.* (15) were the first to show that ultra-violet microscopy might be used to study a single living cell in tissue culture during mitosis. Similar observations have recently been made by Wilkins (14) on mouse fibroblasts. However it has been found (5) that under certain well defined conditions only one or two photographs at 265 mμ of the intermitotic chick fibroblast may be taken if the possibility of altering the metabolism of the cell is to be completely avoided. For this reason, and since it is doubtful whether mitosis is normal under serial u.v. photography, the present studies were restricted to single observations on different cells at various stages of division.

The results have shown that in the living chick fibroblast at metaphase, the total concentration of u.v. absorbing material in the spindle is no greater and probably less than in the surrounding cytoplasm, but that at anaphase, the total concentration of material absorbing at 265 mμ is greater between the chromosome groups than in the adjacent cytoplasm. This agrees with the observations of Jacobson and Webb (7, 8) on fixed material; they found that there was less ribonucleoprotein in the metaphase spindle and more between the anaphase groups than in the neighbouring cytoplasm.

Owing to geometrical uncertainties measurements made on the areas occupied by the metaphase and anaphase chromosomes gave only limits for the amounts of u.v. absorbing material contained in them; for this reason it was difficult to determine whether the extra absorption present between the chromosome groups at anaphase was derived from the chromosomes (7, 8). Attention has already been drawn to the dispersion of the absorbing material in this region in cells damaged by ultra-violet radiation. This may be one reason why it was not reported earlier in u.v. photomicrographs of living cells.

SUMMARY

Ultra-violet photomicrographic studies on mitosis in chick heart fibroblasts *in vitro* are described. Although this technique as applied to living cells is limited by non-specificity, and geometrical difficulties due to over- and under-lying absorbing regions, it can be stated qualitatively that in chick fibroblasts the total concentration of material absorbing u.v. at 265 mμ is not greater and may be relatively less in the spindle at metaphase, and relatively more in the region between the chromosomes at anaphase than in the adjacent cytoplasm.

It is a pleasure to acknowledge the many helpful discussions and encouragements given by Dr. H. B. Fell and Dr. M. H. F. Wilkins. I am indebted also to Mr. P. M. B. Walker for the use of his recording densitometer, to Miss S. Jackson for making the tissue cultures and to Miss F. Ticehurst for preparing the plates.

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CYTOCHEMICAL REACTIONS OF RAM SPERMATOZOA

M. H. G. FRIEDLAENDER and M. J. FRASER

Wheatstone Physics Laboratory and Medical Research Council Biophysics Unit,
University of London, King's College, Great Britain

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THREE main methods are available for the study of the constitution of spermatozoa. Quantitative chemical analysis (18, 34) supplies details of the biochemical composition and metabolism of sperm and plasma, while cytochemical techniques (19, 20, 21, 26, 27) point to the location of the major components. Electron microscope studies (1, 2, 3, 4, 13, 14, 28) yield details of submicroscopic structure and morphology. In order to obtain a more comprehensive picture it is essential to aim at correlating the evidence furnished by a number of methods rather than to rely solely on data supplied by one approach only.

During the course of extensive electron microscope studies of ram spermatozoa[1] it became apparent that little systematic cytochemical information was available for reference purposes. Certain species were investigated in the past by a number of workers, and more recently by Leblond and Wislocki (15, 32). Wislocki's work (33) on the cytochemical reactions of human spermatozoa makes use of more modern histochemical methods. As far as is known, no such systematic investigation has been carried out on ram spermatozoa, where the only cytological study is that by Green (12) which is primarily concerned with the chemistry and cytology of the "sperm membrane."

The work described here aims at applying some of the recent histochemical techniques to the study of ram spermatozoa. The locations of the following substances were investigated: magnesium, calcium, potassium, phosphate, sulphydryl groups, proteins, polysaccharides, nuclear material, lipids, cholesterol, acid and alkaline phosphatase. No attempt was made to deal with the more variable characteristics of semen plasma.

METHODS

The semen was supplied by the laboratory of Dr. A. Walton (Animal Research Station, Cambridge) as described previously (28). High motility was observed. The spermatozoa were washed free of seminal plasma by means of a phosphate buffer (31) which in turn was removed by three rapid washings with glass-distilled water. Smears of the washed sperm were air-dried for sixteen hours.

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[1] To be published separately, but cf. Exptl. Cell Res. **1**, 1 (1950).

A. *Some Inorganic Constituents.* Unless stated to the contrary, smears were fixed with 4 per cent neutral formaldehyde.

Magnesium: Broda's technique (5) was used with 0.2 per cent Titian yellow in alkaline medium. *Calcium:* Von Kossa's silver test for calcium was used as advocated by Gomori (11). Calcium salts reduce added silver salts in the presence of light. *Potassium:* Carere-Comes' Siena Orange method was employed for the detection of potassium (6).

Phosphate: 1) Serra and Queiroz Lopes' (30) modification of the molybdate method for phosphate. The material was fixed in acetic alcohol-formalin (a few drops of glacial acetic acid to 10 ml of a mixture of 2 volumes of 96 per cent alcohol and 1 volume of formalin). 2) A modification of the above method, which differed in the strength of hydrochloric acid (43 instead of 16 per cent) in the molybdate reagent, and the temperature (20° C for 2–3 hours instead of a period of several days at 0° C) was used.

Sulphydryl and Disulphide Groups: 1) Rapkine's Nitroprusside Test. This method comprises three separate tests for free, total and protein-bound sulphydryl groups; appropriate fixatives were used as advised (17).

2) Prussian Blue Test as described by Chèvremont and Frédéric (7). This gave the total sulphydryl content.

B. *Some Protein Reactions.* Two methods, details of which were kindly supplied by J. F. Danielli (8) and L. G. H. Bell, were used for the location of proteins.

1) Dinitrofluorobenzene Method (D.N.F.B.): The basis of this method is a coupling reaction of D.N.F.B. with a specific group in the protein such as a side chain or end group containing a primary amino group, tyrosine (hydroxyl), a carboxylic residue or a sulphydryl linkage. The complex is reduced by sulphur dioxide and a diazonium linkage formed after treatment with nitrous acid. Further coupling with a phenol or aromatic amine is then carried out to intensify the colour developed.

2) Tetrazo-Dianisidine Method: This method is essentially the same as the previous one in which D.N.F.B. was used, except that it is a direct coupling of the protein with tetrazo-dianisidine at 0° C in a buffer solution. The colour is intensified by further coupling with an aromatic phenol or amine.

C. *Polysaccharide Test.* The periodic acid-Schiff procedure was followed as specified by McManus (24). This was recently applied to human sperm by Wislocki (33).

D. *Nuclear Material.* 1) Feulgen Technique. This locates desoxyribonucleic acid by its combination with the reducing sugar residue.

2) Aniline Blue and Orange G: Previous to staining, smears were extracted with 4 per cent trichloracetic acid to remove total nucleic acid. The staining medium was prepared as follows: aniline blue (0.5 g) and orange G (2.0 g) were dissolved in distilled water (100 ml); glacial acetic acid (8.0 ml) was added and the whole brought to the boil and filtered. One part of this solution was used to three parts of distilled water.

Globulins and intranuclear histones retain orange G, whereas aniline blue more slowly stains collagenous and cytoplasmic proteins, including ribonucleoproteins.

E. *Lipids and Cholesterol.* 1) Lipids: (a) Sudan Black: Smears were heat-fixed and stained with a Sudan Black solution (0.3 per cent in 70 per cent alcohol), for 5–15 minutes. After clearing in xylol some smears were counterstained in 0.5 per cent safranin solution for 5–10 seconds and washed in distilled water. (b) Sudan Red: A similar procedure was followed for staining with Sudan Red. (c) Osmium Tetroxide: After heat-fixation, slides were washed in distilled water for 24 hours and subjected to osmium vapour at 37° C for a further 24 hours. They were then washed in running water for 6–12 hours.

2) Cholesterol: Schultz's test (29): This consists of the application of iron alum followed by a mixture of concentrated sulphuric acid and glacial acetic acid.

F. *Acid and Alkaline Phosphatase Activity.* 1) Acid Phosphatase: Smears were fixed in cold acetone for 1 hour at 4° C, in accordance with the technique used by Wislocki (33). Only sodium glycerophosphate was used as substrate, and the reaction was carried out at pH 5, as specified in the revised method of Gomori (9). The location of the enzyme activity was noted at definite time intervals after the addition of the substrate medium.

2) Alkaline Phosphatase: After overnight fixation in cold 80 per cent alcohol at 4° C, sodium glycerophosphate was used as substrate at pH 9.4 (10). The effect of time on the reaction was again noted.

G. *Metachromatic Staining with Toluidine Blue.* Acidic types of compounds, particularly acid mucopolysaccharides, give a purplish coloration with this dye.

For fixation a 4 per cent aqueous solution of basic lead acetate was used, followed by staining with 0.5 per cent aqueous solution of toluidine blue for half an hour.

RESULTS

A. *Inorganic Constituents. Magnesium:* The expected brick-red colour was not observed but instead an orange-yellow reaction was obtained. This method was checked with a pure magnesium salt, and it was confirmed that the characteristic positive reaction was an orange rather than a brick-red coloration. The presence of magnesium was indicated for the whole sperm. The head showed heavier "staining" over the post-nuclear cap and towards the tip of the head. *E* (cf. fig. 1) was particularly heavily outlined, much more so than p_1. As regards the equatorial zone, *e*, it was not clear whether the reaction was just faint or actually negative.

The middle-piece "capsule" (to be discussed later) showed up well and was frequently the site of marked kinks. The middle-piece was found to protrude into the head, well beyond the basal inset of the neck-apex.

Calcium: A greyish colour indicated a positive reaction and was found in the head. It might have been primarily a surface effect which seemed particularly likely in the case of the middle-piece. No positive reaction was obtained for the axial fibre (21, 22). In the head both acrosome and post-nuclear cap took up the silver, the effect being that of a fairly heavily outlined zone surrounding a central, lighter region. *E* was again strongly positive, while *e*

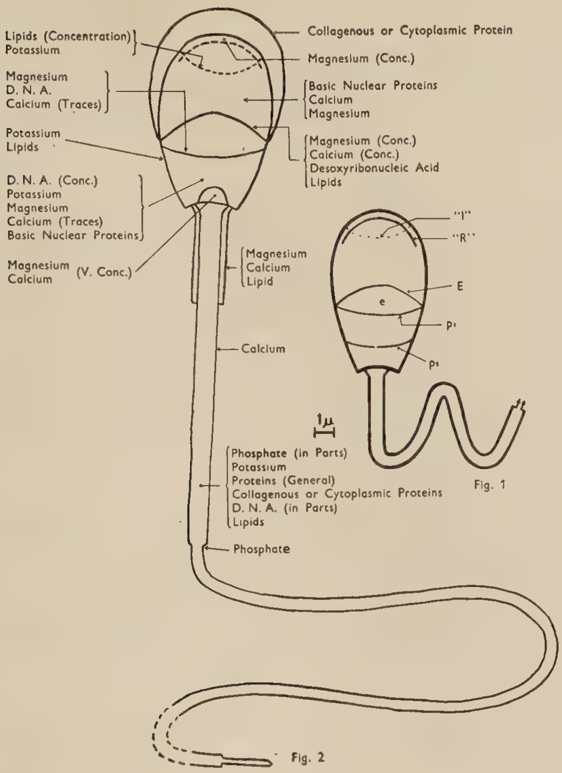
Lipids (Concentration)
Potassium
Magnesium
D. N. A.
Calcium (Traces)
Potassium
Lipids
D. N. A. (Conc.)
Potassium
Magnesium
Calcium (Traces)
Basic Nuclear Proteins
Magnesium
Calcium
(V. Conc.)
Collagenous or Cytoplasmic Protein
Magnesium (Conc.)
Basic Nuclear Proteins
Calcium
Magnesium
Magnesium (Conc.)
Calcium (Conc.)
Desoxyribonucleic Acid
Lipids
Magnesium
Calcium
Lipid
Calcium
Phosphate (in Parts)
Potassium
Proteins (General)
Collagenous or Cytoplasmic Proteins
D. N. A. (in Parts)
Lipids
Phosphate
"I"
"R"
E
e
p₁
p₁
1μ
Fig. 1
Fig. 2

Fig. 1. Reference diagram showing main features in the head.[1]
Fig. 2. Simplified diagram showing location of certain chemical constituents.

gave no reaction; p_1 was not always clearly outlined. Precipitation was particularly heavy just above the head base where a fan-like region spread out from the neck-inset. The middle-piece "capsule" was clearly marked.

According to Marza (21, 22), the reaction is very weak, and localised in the axial thread (human, pig, guinea-pig, rat, dog).

Potassium: The whole of the sperm appeared to take up a yellow colour. In general, the post-nuclear cap stained more heavily, particularly at the boundaries, and inversion effects "*I*" (fig. 1) were emphasized and frequently observed; *e* apparently gave no reaction. In some cases the sperm appeared as if stripped of an outer membrane so that a uniform yellow colour was obtained without any darkening boundary effect, which was especially noticeable in the middle-piece. This is of some interest in view of Marza's comment (19): 'Potassium was found in the lipoid mantle around the head (human, cat), and in the lipoid mantle around the middle-piece (rabbit, pig, guinea-pig, bull).'

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[1] Previously, (28), the symbol *E* was used to denote the nuclear ring underlying p_1, the edge of the post-nuclear cap. This ring is now designated by E'; since E' and p_1 cannot be resolved here, p_1 only has been used throughout this paper.

Phosphate: Considerable difficulty was encountered in the identification of a definite blue colour, due to the thinness of the material. Marza (21, 22), in a schematic representation of mammalian sperm constituents, mentions an intense reaction for the galea capitis, the posterior half of the head, and the middle-piece. The only convincing confirmation was found in the middle-piece where the staining was frequently non-uniform, and irregular striations could be observed, particularly towards the middle-piece—tail transition. Even allowing for the technical difficulty of confirming the expected pale blue coloration it was felt that a positive reaction should have been obtained, considering that the known phosphorus content of the head is of the order of 4 per cent. In view of consistently negative results it was assumed that either insufficient hydrolysis of the organo-phosphate esters was taking place or else diffusion of the phosphate ions was too rapid for the formation of the phosphomolybdate complex.

No difference was observed between ordinary sperm and material that had been subjected to severe temperature shock by drastic cooling.

Sulphydryl and Disulphide Groups: 1) Rapkine's Nitroprusside Test gave very inconclusive results; in no case was a positive reaction observed, as indicated by red coloration. This may be accounted for by the unavoidable diffusion of the reaction products, rather characteristic of this technique. 2) Prussian Blue Test. This gave a positive blue reaction, confirming the presence of sulphur throughout the whole of the sperm. In this it rather differs from Marza's generalised conclusions (21, 22) based on investigations of bull, dog, cat, rabbit, pig, guinea-pig, rat and mouse. He claimed that the presence of sulphur was confined to the middle-piece and the anterior centriole, with only a very weak reaction for the posterior half of the head. While his work did not include ram sperm his actual method was not particularly sensitive; this is further supported by the chemical studies of Zittle and O'Dell (34) on bull spermatozoa, confirming sulphur in the head, middle-piece and tail.

B. *Proteins:* 1) D.N.F.B. The mauve or pink colour characteristic of the protein complex was observed in the head, while the middle-piece and tail gave an inconclusive result.

2) Tetrazo-Dianisidine. The reaction was positive for the whole sperm as indicated by a brownish pink. The head stained uniformly except for the base, where heavier staining might perhaps be accounted for by the greater density and thickness of the head in that region. (This point will be discussed further.)

C. *Polysaccharides.* The periodic acid-Schiff procedure gave a negative reaction both before and after the saliva test for glycogen. This is in agreement with Wislocki's observations on mature human sperm although the presence of polysaccharides has been confirmed in the immature sperm of various species (15, 16, 32). Marza's claim (19–22) to have demonstrated glycogen in the middle-piece, neck and post-nuclear cap boundary should be accepted with reserve as no precaution had been taken to ensure the elimination of seminal plasma, and his results might be due to adsorbed surface material.

D. *Nuclear Material.* 1) *Feulgen:* A striking variation amongst individual sperm heads was found, as regards intensity and extent of the reaction. This particularly concerned the definition of the equatorial zone. Three main groups were distinguished: (a) E well defined (more intense than uniformly pink background) i.e. no differentiation into e or p_1. (b) E well defined. Diffuse and highly convex e; no clear p_1-outline. (c) E well defined, but not by any intensification of colour; e considerably narrowed; clear p_1-outline.

Apart from e-variations, the head stained very strongly at the base, immediately above which there was a fairly transparent zone as was frequently observed also above E. The remaining post-nuclear cap and acrosome portions of the head showed an intermediate degree of staining.

The middle-piece took up the stain over short irregular portions. The "capsule" which appears to be an extra sheath ca. 4 mμ long was again observed immediately below the neck.

2) Aniline Blue and Orange G: Orange G stained the head uniformly whilst the galea capitis, middle-piece and tail stained with aniline blue. The possible presence of collagenous material in the galea capitis is of interest in view of some complex fibrils seen in the galea in some electron micrographs.

E. *Lipids and Cholesterol.* 1) Lipids: (a) Sudan Black. E frequently stained heavily as did the outline of the post-nuclear cap. This marked lateral staining, similar to the effect shown in fig. 9, was also reported by Marza (21, 22). Boundary p_1 was not affected. This rather suggests that it is not the post-nuclear cap itself which contains the lipids but rather some material which is near to, or forms part of, the posterior skeletal structure.

The various characteristic structures[1] below the head apex (cf. fig. 1, "*R*" and "*I*") stained heavily, thus confirming a high lipid content.[2]

The middle-piece reaction confirmed the presence of lipids. In addition, remarkable inhomogeneities were frequently observed which showed irregular alternations of staining and completely non-staining portions. The "capsule" stained heavily.

Counterstained preparations gave a pink head and black middle-piece. (b) Sudan Red. This stain is known to be taken up rather more by triglycerides than is Sudan Black. A positive reaction was obtained for the middle-piece only. (c) Osmium Tetroxide. No additional information was obtained.

2) Cholesterol: The Schultz test for cholesterol proved completely negative for ram sperm. Previous workers (23, 25) have recorded its presence in the tail of salmon sperm but possibly cholesterol is a chemical constituent not found in mammalian sperm.

F. *Acid and Alkaline Phosphatase Activity.* 1) Acid Phosphatase: The presence of acid phosphatase was indicated by a darkening to brown or black where the enzyme was concentrated. Observations were made 1, 3, 6, 14, 24, 36, 48, 72 and 84 hours after the addition of substrate medium.

The most striking effect was a swelling of the head seen after 24 and 36 hours only (figs. 10–14) after which it subsided again. In spite of pronounced individual variations inherent in the material, which must be borne in mind when gauging the effect of any treatment on spermatozoa, certain features emerged, as illustrated in figs. 3–16.

Intense activity was shown by a rim surrounding the tip of the head, and was frequently accompanied by granular deposits (fig. 6). Striations in the anterior part of the head were probably part of the galea capitis (figs. 8, 13 and 15). Generally, the following showed no activity: *e*, a narrow well-defined zone immediately above *E*, and a narrow basal zone.

Swelling appeared to be confined to the part of the head above p_1; the galea capitis could sometimes be distinguished, still attached (fig. 13). The fate of the equatorial zone, *e*, on the other hand, was not at all clear. While *e* may have been actively involved in the swelling process, there were indications to the contrary. The drastic swelling of part of the anterior half of the head might have depressed the equatorial zone, producing a double boundary at the separation of the anterior and posterior portions, as shown in fig. 11.

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[1] Also described in Exptl. Cell Res., **1**, 1 (1950). Plates II d and III b and c.

[2] Marza has described a knob-like structure at the tip of the head, containing lipid material. Since M.H.G.F. has only observed such a structure in abnormal bull sperm, but never in ram, it cannot be ascertained whether the intense blackening of "*R*" described by us for ram might have been mistaken for the structure described quite generally by Marza.

The anterior part would therefore appear as a full circle in the broad-side view.

Chain-like aggregates of globular or granular material showing strong phosphatase activity were often seen in the background. These were previously reported by Wislocki (33, Plate 2, fig. 7). We do not agree that they are due to a plasma component of high enzyme activity but suggest that they are due to material from the sperm head itself. In tracing the occurrence of this background material it was most noticeable where swelling was extreme, i.e. after 24 and 36 hours when some heads had actually burst.

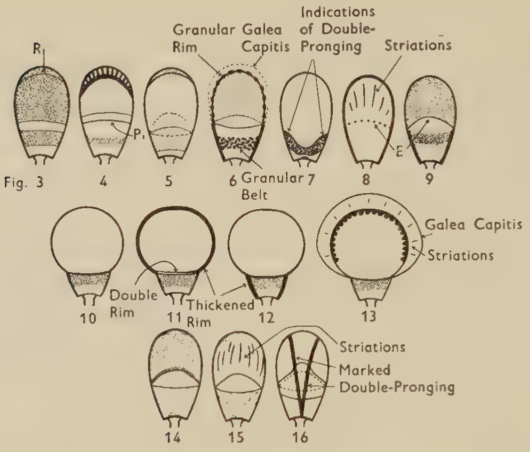
A very intense line was sometimes observed from the tip to the base of the head when viewed in profile. Since this line had occasionally been seen with the head broad-side on, the thickened rim (cf. figs. 11 and 12) could not account for this meridional effect. (Cf. Popa's comments on the central canal through the sperm head (26, p. 105); this included work on ram sperm.) Evidently, considerable time for penetration of the reagents was required since this feature became quite common with increasing duration of reaction.

A frequent twisted appearance of the middle-piece could be accounted for by a gross spiral structure seen during the swelling of the middle-piece, and which sometimes becomes partly detached. The interior stained heterogeneously.

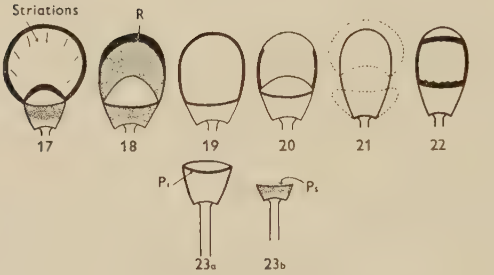
2) Alkaline Phosphatase: A greyish or brown colour indicated the site of alkaline phosphatase activity, and observations were made 1, 3, 6, 24, and 72 hours after the addition of substrate medium. Throughout, the reaction was less well defined than that of the acid phosphatase. Swelling took place rapidly, being at its most pronounced between 1 and 3 hours. On the whole, it was general (e.g. figs. 18 and 19, as compared with 17) although occasionally spermatozoa appeared in which the head cap appeared to have swollen rather than the head proper.

There were signs of marked activity in the anterior rim, which appeared as a partially granular belt, up to 1 mμ in width. It disappeared entirely after 24 hours (cf. fig. 22). No enzyme activity was found in *e*; the alkaline phosphatase distribution for the remainder of the head can be seen in figs. 17–23.

Intense activity in the middle-piece was confined to the "capsule" and Jensen's ring; elsewhere there were occasional breaks in the uniformity of staining. Enzyme activity decreased with increasing distance from the neck, the most active portion being immediately above the inset of the neck into the head (not shown in the diagrams). Heterogeneity of staining increased with time.


R₁
Granular Galea Rim
Capitis
Indications of Double-Pronging
Striations
P₁
E
Fig. 3
4
5
6
Granular Belt
7
8
9
Galea Capitis
Striations
Double Rim
10
11
Thickened Rim
12
13
Striations
Marked Double-Pronging
14
15
16

Acid Phosphatase Activity


Striations
R
17
18
19
20
21
22
P₁
P₂
23a
23b

Alkaline Phosphatase Activity

The following diagrams show the location of acid phosphatase by the Gomori method after varying time intervals.

Figs. 3–7. Representative diagrams showing sites of enzyme activity after 3 hours' incubation.
3. Main zones, with little activity near the tip.
4. Mainly unstained zones, with striations between *R* and tip.
5. Tip stained but no striations.
6. Very coarse granularity where indicated. Occasional slight swelling of galea capitis.
7. First indications of "double-pronged" differentiation in posterior portion.

Fig. 8. 6 hours' incubation. Irregular but marked striations in anterior portion of head.

Fig. 9. 14 hours' incubation. Heavy lateral staining in posterior part. Striations sometimes observed in zone immediately below p_1, indicated here by a darkened band.

Fig. 10. 24 hours' incubation. Swelling in anterior part.

Figs. 11–13. 36 hours incubation.
11. Heavily stained rim for entire anterior portion.
12. Posterior lateral boundary heavily stained.
13. Galea capitis distinct, showing striations. Rim marked with opaque granular aggregates terminating some distance above p_1.

Fig. 14. 48 hours' incubation. Swelling subsided.

Fig. 15. 72 hours' incubation. Striations in upper part of head. Faint indication of rudimentary "double-pronging."

Although it has previously been reported (18) that ram semen has a much lower total phosphatase activity than either human or bull semen, it had also been shown that the bull spermatozoa themselves have an inherent enzyme activity. It is to be expected that if ram spermatozoa have any residual enzyme activity it will be small, and for practical biochemical analysis negligible. It was, however, quite easily detected by cytochemical means, a stronger reaction being obtained for the acid than for the alkaline phosphatase. The marked individual differences in any one preparation might adequately be accounted for by differences in the relative ages of the spermatozoa. The term "age" here refers to the time which has elapsed since the particular spermatozoon has attained maturity, with a corresponding change in biochemical balance of the enzyme systems. At present, all such suggestions must remain hypothetical.

Granularity becoming more pronounced after long periods of incubation might be due to the aggregation of colloidal cobalt sulphide particles. A disperse colloidal system tends to form larger aggregates after long periods in contact with charged ionic particles or charged surface material. Thus many observed locations of enzyme activity may be seen as accumulations in either granular or striated form, instead of a more diffuse reaction.

The swelling observed in the acid and alkaline phosphatase incubations occurred in all the spermatozoa after a certain time, and subsided again. Other workers (33) unfortunately did not record observations of enzymic activity during this swelling period. The general significance of this, presumably osmotic, phenomenon cannot be gauged without further investigation.[1]

Fig. 16. 84 hours' incubation. Shift in E and p_1 demarcation from original position, marked as dotted lines. V-marking from base across the head. Diagram merely indicates outline.

The following diagrams show the location of alkaline phosphatase after varying time intervals.

Figs. 17–19. After 1 hour's incubation.
  17. Marked swelling with heavy staining of boundaries. Striations were observed.
  18 and 19. Drastic but more general swelling of the head. Diagrams indicate extremes of zonal differentiation.
Figs. 20 and 21. 3 hours' incubation.
  20. Some swelling still present. Activity mostly confined to boundaries.
  21. Exaggerated type of swelling occasionally observed.
Figs. 22 and 23 a and b. 6 hours' incubation.
  22. Sharply defined enzymic zones. No activity near tip.
  23 a and b. Head fragments, with breaks at p_1 and ps.

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[1] Popa (25) reports drastic swelling of the galea capitis in the female genital tract, leading to ultimate disintegration.

DISCUSSION

On the basis of our observations the location of various constituents can be represented diagrammatically, as shown in fig. 2. This is largely self-explanatory but certain components are not included as their distribution is too widespread. This applies to sulphur which is present uniformly throughout the whole of the sperm. Similarly, a general protein reaction was observed for the whole of the head (8), with a concentration near the base. Basic nuclear proteins were confirmed throughout the whole of the head (section D) as was desoxyribonucleic acid. In the case of the latter, however, there was some differentiation, the posterior part staining more intensely whilst little reaction was obtained for the equatorial segment.

Any rigid conclusion regarding the location of phosphatase activity is seriously hampered by the great variation in distribution with time, which is not understood. However, there can be little doubt that there is a concentration of acid phosphatase near the tip of the head, and in positions corresponding to the various cap boundaries. Both the basal zone and the equatorial segment are largely free from phosphatase activity.

It is frequently difficult to determine what ought to be considered a mere surface effect. This is particularly true for calcium. In previous work, especially that of Marza and Popa (27), the technique for the separation of sperm from semen is inadequate. Also, the claim for high concentration of constituents near the base of the head seems to have been made rather too freely; electron micrographs have shown considerable thickening of the head immediately above the base, in addition to a dense network of reinforcing fibrils. This might give rise to misleading optical effects. We also feel that the high chemical complexity attributed to the "anterior centriole" is not a strictly true description. The region referred to is not situated below but above the neck-apex and its inset into the head, and the meaning of the work "centriole" in this sense becomes rather obscure. Although both Marza and Popa have included ram among the animal sperm investigated by them, no specific mention is made of their results beyond the fact that the lipid mantle said to surround the head is missing in ram sperm.

On referring to fig. 2 it will be seen that the galea capitis contains collagenous or cytoplasmic proteins. The anterior portion of the head contains calcium, potassium and magnesium, with a concentration of magnesium near the tip. Markings near the tip show a high lipid content, as well as considerable amounts of potassium. Boundary *E* is very rich in magnesium, calcium, desoxyribonucleic acid and lipid. The equatorial segment, on the other hand, shows little evidence of any of these constituents; p_1 contains magnesium, with traces of D.N.A. and calcium. Traces of calcium have also been confirmed in the posterior half of the head which contains potassium and magnesium. Its D.N.A. content is very high. The lateral boundaries are rich in potassium, and even more so in lipid material. The basal zone shows concentrations of D.N.A. and proteins. The small, often well-defined region immediately above the neck-inset into the head, while containing most of the previously mentioned constituents, is particularly rich in calcium and magnesium.

The middle-piece is remarkable for certain gross heterogeneities in staining reactions (not shown). Some portions (ca. 1–3 μ in length) give a positive reaction, while others equally irregular in size and position give no reaction at all. This is particularly noticeable in the case of D.N.A. by the Feulgen method, the phosphatase reaction, and the response to the lipid tests. Unfortunately, no evidence is available which might correlate these three results as regards relative positions. The presence of general proteins and those of a collagenous or cytoplasmic type were confirmed, as well as potassium and some surface calcium.

The "capsule" has a high lipid content besides containing calcium and magnesium. Towards the middle-piece—tail transition the phosphate reaction becomes rather more pronounced.

In general, no reliable information was obtained for the tail as the reduction in width to ~ 0.5 mμ makes the identification of the results of certain reactions rather difficult.

SUMMARY

An investigation into the location of certain chemical constituents in the mature ram spermatozoon is described. This included magnesium, calcium, potassium, phosphate, sulphydryl groups, proteins, polysaccharides, nuclear material, lipids, cholesterol, and acid and alkaline phosphatase.

The relative merit and significance of the results are discussed, and the distribution of the major constituents summarized diagrammatically.

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CROSS FERTILIZATION WITH THE SPERM OF *ARBACIA LIXULA*

CLIFFORD V. HARDING[1] and DRUSILLA HARDING

The Wenner-Gren Institute, University of Stockholm, Sweden, and the Zoological Station, Naples, Italy

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THE cross fertilization of certain marine eggs with the sperm of *Arbacia lixula* is ordinarily very difficult or impossible to obtain. It is known, however, from the experiments of Elster (1, 2) and Hultin (3), that the addition of a small amount of *Arbacia* egg water to the fertilization medium can effect some small percentages of cross fertilization.

During the course of a more general investigation it was found that a combination of *Arbacia* egg water plus either glycine or sodium periodate can greatly increase the capacity of *Arbacia* sperm for cross fertilization. The present paper consists of a description of this effect with the eggs of *Paracentrotus lividus*. The quantities of cross fertilized eggs made available by this procedure would be sufficient for a variety of biochemical investigations on hybrid development.

METHODS AND MATERIALS

Specimens of *Arbacia lixula* and *Paracentrotus lividus*, obtained in the Bay of Naples, were kept in the laboratory aquaria until used. On several occasions it was found that *Paracentrotus* which had been stored in the aquaria for about a week had larger quantities of *fertilizable* eggs than freshly obtained animals. As a rule, therefore, the *Paracentrotus* were stored in the aquaria for several days before being used.

To obtain the eggs or sperm from *Paracentrotus*, the animal was first washed externally with sea water and then the oral side was cut away. After removal of the digestive tract, the interior was washed with sea water to remove any debris and the gonads were then placed in small glass dishes. Testes were allowed to shed into dry dishes. The concentrated sperm was kept in a test tube in the refrigerator until used. The ovaries were placed in sea water until the majority of eggs had been shed. The eggs were then strained through cheese cloth to remove bits of ovarian tissue, and then washed three times in sea water.

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[1] National Institutes of Health Postdoctorate Fellow.

In the case of *Arbacia*, the animals were induced to shed by injecting 0.5 to 1.0 ml of isotonic KCl (0.53 M) into the oral opening. Sperm were shed into dry dishes and eggs into sea water. The subsequent treatment of eggs and sperm was essentially the same as in the case of *Paracentrotus*.

It was found best to adopt a standard method for the preparation of *Arbacia* egg water. A heavy suspension of *Arbacia* eggs was washed once with sea water; after the eggs had settled, the supernatant was discarded. A 10 per cent suspension of eggs in sea water was then prepared and allowed to stand for from 2 to 6 hours with frequent gentle mixing. The supernatant was then decanted and filtered.

All solutions were made up in filtered sea water. Measurements of pH were made with a Beckman pH meter. The barium salt of ATP (Sigma Co. preparation) was dissolved in dilute HCl, and converted to the sodium salt by the addition of sodium sulfate. After centrifugation the supernatant was poured off, diluted to the appropriate concentration with sea water, and the pH adjusted to neutrality with dilute NaOH.

A typical experiment was carried out in the following manner. Syracuse dishes, each containing a final volume of three ml were used. A drop of *Paracentrotus* eggs was placed in the experimental solution, allowed to stand for 10 minutes, and then *Arbacia* sperm were added (two drops of "dry" sperm were added to one ml of sea water, and two drops of this suspension were then added to each dish). Shortly after sperm addition, the eggs in each dish were examined for fertilization membranes, and, at a later time, for cleavages. For each examination 100 eggs were observed, and the number which had elevated fertilization membranes or cleaved was recorded. Unless otherwise stated, the fertilization membranes and cleavages were normal.

RESULTS

Experiments were set up, according to the procedure described above, to test the effect of egg water, alone and in combination with glycine, on the cross fertilization of *Paracentrotus lividus* by *Arbacia lixula*. Whereas very little or even no cross fertilization was obtained in the presence of egg water alone (an average of 0.7 per cent cross fertilization in 26 experiments), a noticeable improvement could be obtained with a combination of egg water plus glycine (final conc., 1.3×10^{-2} M). Moreover, glycine alone had much less effect than a combination of egg water plus glycine. Crystalline serum albumin was found to have a similar effect.

Fig. 1 represents the results of a single experiment in which various concentrations of egg water were used in the presence of 1.3×10^{-2} M glycine. The per cent cross fertilization increased with increasing concentration of egg water from a value of only 2 per cent in the absence of egg water up to a value exceeding 90 per cent. The shape of this curve varies somewhat with different preparations of egg water. In some cases, as, for example, in Fig. 2, curve A, the per cent cross-fertilization increases, goes through a maximum,

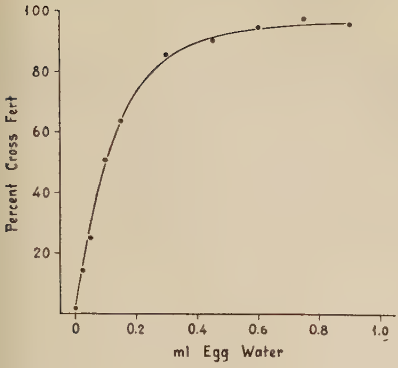

Fig. 1.

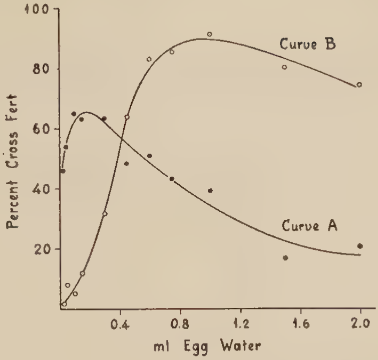

Fig. 2.

Fig. 1. Per cent cross fertilization in various concentrations of egg water plus glycine (final concentration 1.3×10^{-2} M). The final volume of the fertilization medium in all cases was 3 ml.

Fig. 2. Curve A: Per cent cross fertilization in various concentrations of egg water plus glycine (final concentration 1.3×10^{-2} M).

Curve B: Per cent cross fertilization in various concentrations of egg water plus $NaIO_4$ (final concentration 6.7×10^{-5} N) *and* glycine (final concentration 1.3×10^{-2} M).

The final volume of the fertilization medium in all cases was 3 ml.

TABLE I

Per cent cross fertilization in the presence of different combinations of egg water and glycine. Final volume of the fertilization medium was, in each case, adjusted to 3 ml with sea water.

Glycine concentration	ml Egg Water					
	0.025	0.10	0.30	0.60	1.00	2.00
2.3×10^{-3} M	0	0.5	1	1	0	6
4.4×10^{-3} M	3	29	6	65	69	57
8.9×10^{-3} M	41	75	88	91	97	53
17.7×10^{-3} M	80	91	80	94	91	86

and then gradually decreases with increasing concentration of egg water. This inhibiting action of higher concentrations of egg water has been reported earlier by Elster (2).

The effect of various combinations of egg water and glycine on the cross fertilization of *Paracentrotus* by *Arbacia* is indicated in Table I. The per cent cross fertilization, at all the concentrations of egg water used, is increased with increasing concentration of glycine.

In addition to glycine, two other amino acids, α-alanine and β-alanine were used. Solutions (of pH 7.7) were tested in the usual manner, with dif-

TABLE II

Comparative effects of α-alanine, β-alanine, and glycine on cross fertilization in the presence of *Arbacia* egg water. For each determination, a series of six different concentrations of egg water was used. The highest per cent cross fertilization is recorded for each determination.

% cross fertilization of eggs in egg water	% cross fertilization of eggs in egg water + 1.49×10^{-2} M α-alanine	% cross fertilization of eggs in egg water + 1.49×10^{-2} M β-alanine	% cross fertilization of eggs in egg water + 1.77×10^{-2} M glycine
2	18.5	0	98
0	6	1	34

ferent egg water concentrations. β-alanine showed no significant activity: α-alanine, a little activity; and glycine, in control experiments, was very effective (see Table II).

The effect of sodium periodate on the activity of Arbacia egg water. According to Immers and Vasseur (4) the oxidation of *Echinus esculentus* jelly coat solution (concentrated egg water) with $NaIO_4$ abolishes its agglutinating action on spermatazoa. Runnström and Kriszat (5) have found that the addition of $NaIO_4$ to a sea water solution of *Psammechinus miliaris* jelly coat can rapidly remove its capacity to inhibit elevation of the fertilization membrane. Does $NaIO_4$, then, destroy the action of *Arbacia* egg water on cross fertilization? In order to test this, solutions of egg water were treated with $NaIO_4$ (6.7×10^{-5} N $NaIO_4$ in sea water; pH, approx. 7.5–7.9; temp. approx. 20° C) for 10 minutes, and then used in combination with glycine as the fertilization medium. Control experiments were carried out in the same manner, except that $NaIO_4$ was not added. The results are presented graphically in Fig. 2. Curve A represents the results with untreated egg water plus glycine; and curve B, egg water treated with $NaIO_4$ plus glycine. It can be seen that the maximum of curve B occurs at higher concentrations of egg water than the maximum of curve A. This experiment was carried out many times, always with essentially the same result. Moreover, the capacity of the egg water to cause sperm agglutination was decreased but *not* completely abolished. There has, thus, been some inactivation of the egg water. However, that this is not the only action of $NaIO_4$ is shown by the following experiments in which $NaIO_4$ alone, i.e., without the presence of glycine, proved to markedly increase the activity of certain concentrations of *Arbacia* egg water in facilitating cross fertilization. Here again, the results are quite dependent on the concentrations of egg water and $NaIO_4$. Table III indicates the results of an experiment in which various concentrations of $NaIO_4$ were

TABLE III

Per cent cross fertilization in the presence of different combinations of egg water and sodium periodate. Final volume of the fertilization medium was, in each case, adjusted to 3 ml with sea water.

$NaIO^4$ concentration	ml Egg Water					
	0.025	0.10	0.30	0.60	1.00	2.00
1.67×10^{-5} N	3	17	36	72	18	60
3.3×10^{-5} N	1	6	37	89	94	93
6.7×10^{-5} N	5	2	20	85	98	98
13.4×10^{-5} N	1	1	1	16	90	94

used in combination with different amounts of egg water. It is apparent that with small amounts of egg water (0.1 ml and 0.3 ml) the activity of the egg water is enhanced at lower concentrations of $NaIO_4$, and removed at higher concentrations of $NaIO_4$. With larger amounts of egg water (1.0 ml and 2.0 ml), no inactivation occurred with the concentrations of $NaIO_4$ used.

The effect of pretreatment of the gametes with glycine or sodium periodate. In order to test the effect of pretreatment of the eggs or sperm with glycine, the following experiments were carried out. Eggs were pretreated for 10 minutes with 2.66×10^{-2} M (Table IV, exp. A) or 6.66×10^{-2} M (Table IV, exps. B, C, and D) glycine in sea water. Two drops of these eggs were than placed in three ml of fertilization medium, containing 1 ml of egg water and 2 ml sea water. In the control experiment (Table IV, column 1) untreated eggs were placed in a fertilization medium of the same composition, and two drops of glycine were added (to compensate for the glycine added together with the eggs in the experiment referred to in column 4). Ten minutes later *Arbacia* sperm were added. The results, listed in Table IV, column 4, indicate that glycine pretreatment of the eggs does not produce the full effect (in terms of per cent or quality of cross fertilization, i.e., fertilization membranes were low, and cleavage abnormal) which is to be seen in the eggs fertilized in the presence of both egg water and glycine. Compare column 4 with column 2. However, some of these pretreated eggs did show greater percentages of fertilization than *un*pretreated eggs (column 1).

In further experiments, *Arbacia* sperm were treated for ten minutes with glycine. In experiment A, two drops of "dry" sperm were diluted to one ml

TABLE IV

Effect of pretreatment of the gametes with glycine on the per cent cross fertilization. Figures represent the per cent of eggs with fertilization membranes (fm) and the per cent cleavage (c). In columns 2, 5 and 6 the final concentration of glycine in the fertilization medium was 1.77×10^{-2} M.

	1		2		3		4		5		6	
	Untreated eggs, untreated sperm fert. in egg water		Untreated eggs, untreated sperm fert. in egg water + glycine		Untreated eggs, glycine-treated sperm fert. in egg water		Glycine-treated eggs, untreated sperm fert. in egg water		Untreated eggs, glycine-treated sperm fert. in egg water + glycine		Glycine-treated eggs, untreated sperm fert. in egg water + glycine	
No. of Exp.	fm	c	fm	c	fm	c	fm	c	fm	c	fm	c
A	0	3	94	95	0	9	0	0	85	85		
B	0	8	88	84	0	9	24[1]	36[2]	72	79		
C	0	4	98	95	0	6	40[1]	50[2]	73	85		
D							29[1]	35[2]			75	79

with 2.66×10^{-2} M glycine; in experiments B and C one drop of "dry" sperm was diluted to one ml with 6.66×10^{-2} M glycine. Two drops of these treated sperm were then added to *Paracentrotus* eggs in the presence of 1 ml of egg water and 2 ml of sea water. In the control experiments, corresponding amounts of sperm were diluted to 1 ml with sea water, allowed to stand 10 minutes, and then, 2 drops of this suspension and 2 drops of glycine were added simultaneously. The results (Table IV, column 3) indicate that pretreatment of the sperm in this manner does not increase their cross fertilizability to the extent which occurs when cross fertilization is carried out in the presence of both egg water and glycine (compare column 3 with column 2). That the gametes were not injured by the pretreatment procedure is indicated by the fact that they were still capable of high percentages of cross fertilization, when both egg water and glycine were present in the fertilization medium (see columns 5 and 6).

Experiments in which the gametes were pretreated with $NaIO_4$ were also carried out. The procedure was essentially the same as that in the experiments with glycine. The details of the procedure are recorded along with the tabulated results. Eggs were pretreated with 6.7×10^{-5} N $NaIO_4$ for various lengths of time, washed twice with 100 ml of sea water and then placed in a fertilization medium consisting of 1.6 ml of egg water and 1.4 ml of sea

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[1] fertilization membranes very low.

[2] abnormal divisions, arrested development.

TABLE V

Effect of pretreatment of the eggs with 6.7 × 10^{-5} N sodium periodate on cross fertilization. Figures represent the per cent cleavage of eggs treated as indicated at the top of each column. In columns 2 and 4 the final concentration of sodium periodate in the fertilization medium was 6.7 × 10^{-5} N.

1	2	3	4	
Untreated eggs fert. in egg water	Untreated eggs fert. in egg water + $NaIO_4$	$NaIO_4$-treated eggs fert. in egg water	$NaIO_4$-treated eggs fert. in egg water + $NaIO_4$	Length of pretreatment of eggs with $NaIO_4$
0	99	41	98	60 mins.
		20	95	30 mins.
		26	99	5 mins.
0	93	4	79	60 mins.
		1	57	10 mins.

water. Ten minutes later, one drop of *Arbacia* sperm was added. These pretreated eggs did not show as high percentages of fertilization as eggs fertilized in the presence of egg water plus $NaIO_4$ (Table V, compare column 3 with column 2), although they did show higher percentages of fertilization than unpretreated eggs fertilized in the presence of egg water alone. Column 4 of Table V indicates that the pretreated eggs were still capable of relatively high percentages of cross fertilization when the fertilization medium contained both egg water and $NaIO_4$. *Arbacia* sperm, pretreated for 11 or 15 minutes with $NaIO_4$ (6 drops of "dry" sperm diluted to 3 ml with 6.7 × 10^{-5} N $NaIO_4$) were added to *Paracentrotus* eggs in the presence of 2 ml of egg water and 1 ml of sea water. In control experiments 6 drops of "dry" sperm were diluted to 3 ml with sea water and allowed to stand for the same length of time before addition to the eggs. The pretreated *Arbacia* sperm showed no increased capacity for cross fertilizing *Paracentrotus* eggs in the presence of *Arbacia* egg water, although they were still capable of cross fertilizing in the presence of both egg water and $NaIO_4$ (Table VI, compare column 3 with column 4).

The activity of Arbacia egg water at different pH values. It has been previously reported that *Arbacia* egg water is more effective in facilitating cross fertilization in the alkaline pH range (1) and this has been confirmed in the present experiments. It thus becomes of importance to differentiate between

TABLE VI

Effect of pretreatment of the sperm with 6.7×10^{-5} N sodium periodate on per cent cross fertilization. Figures represent the per cent cleavage of eggs fertilized with sperm treated as indicated. In the experiments referred to in columns 2 and 4 the final concentration of sodium periodate in the fertilization medium was 6.7×10^{-5} N.

1	2	3	4	
Untreated sperm fert. in egg water	Untreated sperm fert. in egg water + $NaIO_4$	$NaIO_4$-treated sperm fert. in egg water	$NaIO_4$-treated sperm fert. in egg water + $NaIO_4$	Length of pretreatment of sperm with $NaIO_4$
0	1	0	15	15 mins.
0	18	0	35	11 mins.

TABLE VII

Per cent cross fertilization in the presence of egg water (0.6 ml diluted to 3 ml) at a series of different pH values. For comparison, the effects of egg water plus glycine (final conc., 1.77×10^{-2} M) and egg water plus $NaIO_4$ (final conc., 6.7×10^{-2} N) are included.

1	2	3	4
pH of fert. medium	Fert. in egg water	Fert. in egg water plus glycine	Fert. in egg water plus $NaIO_4$
8.7	42		
8.3–8.4	0		
8.2–8.3	2		
7.5–7.9	0	98	97
7.2–7.4	0		

the effect of a substance, such as glycine or sodium periodate, and the effect of pH change alone. The pH of all solutions of glycine or $NaIO_4$ in sea water, used as fertilization media, was approximately 7.5 to 7.9. Control experiments, in which egg water alone, at the same pH, was used as the fertilization medium, showed very little or no activity. However, by increasing the pH to 8.7, the egg water alone became considerably more effective in facilitating cross fertilization. Table VII, column 2, demonstrates the differences in activity of egg water at each of a series of different pH values. There is no significant activity below pH 8. For comparison, the activity of the egg water in the presence of glycine (column 3) and also in the presence of $NaIO_4$ (column 4) is included. The glycine-egg water solutions as well as the $NaIO_4$-

egg water solutions had pH values of approximately 7.5–7.9. It would seem, therefore, that the glycine and $NaIO_4$ have their effects independently of alkaline pH.

It is perhaps of some interest that both glycine and $NaIO_4$ have, in certain cases, been reported to improve homologous fertilization (5, 7, 8). Adenosine-triphosphate (ATP) has also been reported to improve homologous fertilization in the eggs of *Psammechinus miliaris* (6). Accordingly, several experiments were carried out to test the effect of ATP on cross fertilization. ATP (2.67×10^{-3} M, final concentration) was used in combination with egg water of different concentrations. Although the per cent cross fertilization was increased in the presence of ATP in several experiments, this was not always the case. The results appear to depend to a great extent on pH. At a pH slightly below that of sea water there was no significant effect. At higher pH values, a significant per cent cross fertilization could be obtained in the presence of ATP, but in some of these experiments high percentages occurred also in the controls. It is thus questionable whether ATP has a specific action in this case.

DISCUSSION

Elster (1, 2) and Hultin (3) have shown that *Arbacia* egg water can produce some increase in the capacity of *Arbacia* sperm for cross fertilization. The egg waters of other species of sea urchin were without effect (3). In the present investigations, the jelly coats were not removed from the *Paracentrotus* eggs, which means that *Paracentrotus* egg water should have been present in all the experiments referred to. In spite of this, however, cross fertilization was not obtained in any great amounts unless *Arbacia* egg water was present in the fertilization medium. Egg water alone is very ineffective. However, the combination of *Arbacia* egg water and a non-specific substance such as albumin (cf. also Hultin, (3)), glycine, or $NaIO_4$ can strikingly increase the per cent cross fertilization. Glycine or $NaIO_4$ alone are also very ineffective. Pretreatment of the gametes with either glycine or $NaIO_4$, followed by cross fertilization in the presence of egg water results in lower percentages of cross fertilization than in the case in which both egg water and glycine or $NaIO_4$ are present in the fertilization medium.

For practical purposes, in obtaining high percentages of cross fertilized eggs, it is perhaps easiest to use a combination of egg water plus a relatively high concentration of glycine (about 1.8×10^{-2} M). At this concentration of glycine, high percentages of cross fertilization can be obtained over a relatively wide range of egg water concentration (see Table I). A knowledge of the exact mechanism, whereby the specifically active *Arbacia* egg water plus glycine or $NaIO_4$ interact with sperm and egg to produce this increased capacity for cross fertilization, must await further investigation.

SUMMARY

1. Experiments on cross fertilization were carried out with the eggs of *Paracentrotus lividus* and the sperm of *Arbacia lixula*. The per cent cross fertilization was greatly increased when the fertilization medium contained a mixture of *Arbacia* egg water and glycine. Egg water alone or glycine alone had much less effect. Crystalline serum albumin had an effect similar to that of glycine. α-alanine was also effective, but β-alanine had no effect at the concentrations used.

2. A combination of certain low concentrations of sodium periodate and *Arbacia* egg water also increased the per cent cross fertilization. Higher concentrations of sodium periodate inactivated the egg water.

3. Pretreatment of the gametes with either glycine or $NaIO_4$, followed by fertilization in the presence of egg water, did not result in as high percentages of cross fertilization as in the case of fertilization in the presence of glycine and egg water or sodium periodate and egg water.

4. Very little cross fertilization occurred in the presence of egg water alone at a pH of about 8 or below. At a more alkaline pH (8.7), a relatively high percentage of cross fertilization occurred. A combination of egg water plus glycine or egg water plus sodium periodate increased the per cent cross fertilization at pH values below 8, indicating that this effect is independent of alkaline pH. A combination of egg water and ATP did not increase the per cent cross fertilization at pH values below 8.

We wish to express our sincere appreciation to Professor John Runnström for originally suggesting this problem and for all his help and guidance. We should like also to thank Professor R. Dohrn, Dr. A. Monroy, and the staff of the Zoological Station, Naples, for making our stay there a pleasant one.

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BRIEF NOTES

UEBER EINIGE EIGENSCHAFTEN REKONSTITUIERTER KOLLAGENER FASERN

G. F. BAHR

Institut für Zellforschung, Karolinska Institutet, Stockholm, Schweden

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Wie schon in einer vorläufigen Mitteilung erklärt wurde (1), lassen sich von Rattenschwanzsehnen, welche in 1: 25 000 Essigsäure suspendiert werden, klare, visköse Lösungen abfiltrieren. Zuerst vorsichtiges Ablaufen lassen durch doppelte Baumwollgaze, dann Absaugen durch Glasfiltertiegel mit jeweils kleiner werdender Porenweite. Bei etwa 5 μ Porenweite lösen dann die kapillaren Räume eine vorzeitige Präzipitation des Kollagens und Verstopfung des Filters aus. Auch haften sehr kleine Kollagenpartikel fest an Glasoberflächen (11). Schliesslich werden die Lösungen bei 3 500 Touren für 15 Min. zentrifugiert und bleiben — an kalter Stelle — für 2–3 Tage aufgehoben unverändert. Zur Prüfung der Lösungen gibt man einen kleinen Tropfen auf ein Objektglas und bedeckt ihn mit einem Deckglas, dessen Ecken mit Paraffinwülsten versehen sind. Dann wird ein Tropfen 1 % NaCl an den Rand des Deckglases gesetzt, der den Kollagentropfen sofort umschliesst. So kann die langsame Präzipitation der Kollagenfibrillen im Dunkelfeld beobachtet werden.

Die Rekonstitution kann, ausser in gewöhnlichen Dialyseschläuchen, auch in kleinen Kammern vorgenommen werden, deren eine Abb. 1 zeigt. Wird die Dialysekammer von den EM-Nickelnetzen entfernt, so kann mit etwas aqua dest. das überschüssige Material entfernt werden. Die zurückbleibenden, festhaftenden Kollagenfibrillen liegen daher so, wie sie entstanden sind. Hierdurch werden Lagemissdeutungen der Fibrillen zueinander eingeschränkt. Dieselbe Anordnung wurde in vergrössertem Masstab verwendet um mit Hilfe vergleichender Photometrie (Pulfrich) den Einfluss von Temperatur, Salzkonzentration, Konzentration der Kollagenlösungen und der Jonenkombination zu erfassen. Sind die Fibrillen nach 24 Std. präzipitiert, was an einer deutlichen, milchigen Trübung zu erkennen ist, so werden die Kammern oder Schläuche für 2 × 24 Std. gegen aqua dest. gesetzt um überschüssiges Salz zu entfernen. Der Inhalt der Dialyseschläuche (etwa 5 ccm) wird mit einem langsam laufenden Blendor in 20 ccm aqua dest. suspendiert und mit einer — stets frischen — Pipette auf die vorbereiteten EM-Objektträger aufgebracht. OsO_4 und Phosphorwolframsäure werden zur Kontrasterhöhung verwandt. Ausserdem werden noch zwei andere Methoden zur Fibrillenrekonstitution angewandt: Ein Tropfen Kollagenlösung auf das vorbereitete EM-Netz, Bedecken mit feuchter Dialysemembran, darauf ein Tropfen Salzlösung, nach 20 Min. Abziehen der Membran. Es bleiben genug Fibrillen auf dem Netz zur Beobachtung. Oder: der Kollodiumfilm wird direkt auf einer entsprechenden Salzlösung erzeugt, darauf die EM-Netze gesetzt und mit Kollagenlösung besprüht, Bedecken mit Glasglocke um das Austrocknen des aufgesprühten Kollagens zu vermeiden, Fischen der Netze mit einem Objektträger in der üblichen Weise.

Die rekonstituierten Kollagenfibrillen können in allen Typen und Formen im EM beobachtet werden wie sie auch normales Gewebe zeigen (2–10). Doch muss betont werden, dass die feinere Struktur und die chemische Zusammensetzung, deren Beurteilung entweder durch das Auflösungsvermögen des EM begrenzt ist oder besonderer chemischer Methoden bedarf, Abweichungen vom Normalen zeigen könnte. Die rekonstituierten Kollagenfibrillen haben Dicken von 1 000–1 200 Å bis herunter zum Auflösungsvermögen des EM. Die gewöhnliche Periode der Kollagenfibrille wurde zu 635 Å bestimmt. Dieses durch vergleichende photometrische Messungen entlang der Fibrillen und über Polyvenyl-Latex-Kugeln (260 mμ). An besonders schmalen Fibrillen wurden Perioden von 400 Å herunter bis zu 190 Å mit einem Durchschnitt von 210 Å gemessen. Doch scheinen diese Variationen Artefakte zu sein, denn sobald dieselben schmalen Fibrillen sich gröberen Fibrillen anlegten, entsprachen die Periodenbänder den bekannten Unterbändern der 635 Å-Periode. 635 Å/3 = etwa 210 Å.

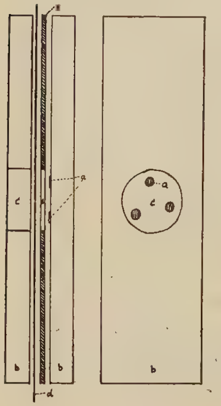


Abb. 1.

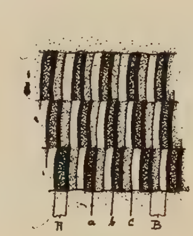


Abb. 2.

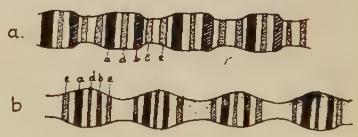


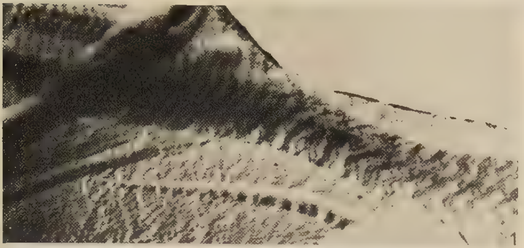
Abb. 3.

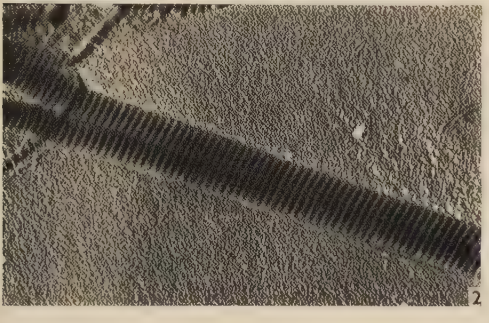
Abb. 1. Reissen des Kollodiumfilms. Die grosse Periode splittert in die Unterbänder auf. Pt/Pd beschattet. Ba-acetat.

Abb. 2. Mg-citrat, pH 4,1, Pd beschattet. Zusammenlaufen zweier Fibrillen. Perioden im Register.

Abb. 3. Ni-sulfat, pH 4,0. Pd beschattet. Phosphorwolframsäure-Imprägnation.

Reisst der Kollodiumträgerfilm während der EM-Beobachtung, so können besondere Eigenschaften der rekonstituierten Kollagenfibrille zur Darstellung kommen. Die Hauptperiode kann dabei durch Druck in axialer Richtung auf 400 Å vermindert werden, wobei die Fibrille an Dicke zunimmt. Dehnung ist bis 1 500 Å möglich. Am besten zu beobachten an unbehandeltem, unbeschattetem Material. Fig. 2 gibt schematisch die ungefähre Massenverteilung (Dichte) entlang der Fibrille wieder.





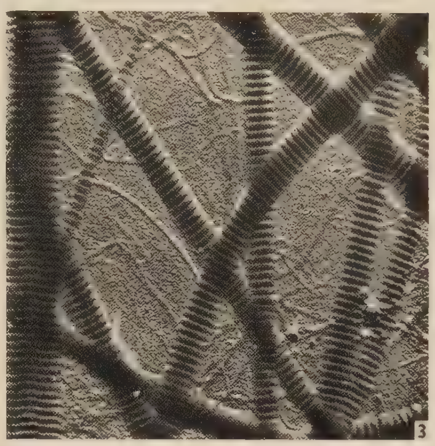


Fig. 1. Kleine Dialysekammer, Objektträgergrösse. a = EM-Netze, b = Objektträger, c = Loch im Objektträger und Glimmerplätchen, d = Dialysemembran, e = Glimmerplättchen. c wird mit Kollagenlösung gefüllt.
Fig. 2. Schema der Intensitätsverteilung entlang der Fibrille.
Fig. 3. Bandmuster bei leicht (a) und stärker (b) gedehnten Fibrillen.

Reisst die Fibrille, so geschieht dies entweder in der Zone der geringsten Dichte oder aber unter Aufsplitterung in die drei Unterbänder, die die Hauptperiode ausmachen (Abb. 1). Leichte Streckung der Fibrille offenbart 5 Unterbänder, deren Zuordnung Fig. 3 wiedergibt. Es wurden keine weiteren Unterbänder beobachtet, obwohl die Photometerkuven (1) das Vorhandensein von 7 Unterbändern anzeigen. Einige Zerreissbilder lassen vermuten, dass die Elastizität oder Dehnbarkeit der einzelnen Bänder in querer Richtung grösser ist als in axialer. Nach diesen Kurven haben die Fibrillen einen ellypsoiden Querschnitt ohne Besonderheiten. Die Fibrillen können in der Längsrichtung aufspalten oder in Büscheln auslaufen, auf deren Einzelfasern die Perioden noch ein Stück zu verfolgen sind. Lagern sich die Fibrillen längs aneinander (Abb. 2 u. 3) so sind die Hauptperioden im Register, oder so gegeneinander verschoben dass die Verknüpfung über die Unterbänder erfolgt, die ineinander übergehen. Fig. 2.

Eine grosse Zahl Bilder zeigt deutlich, dass dieses Prinzip auch unter Verbiegung der Bänder beibehalten wird (Abb. 1).

Die zur Präzipitation verwendeten Jonenkombinationen haben keinen Einfluss auf die 635 Å Hauptperiode. Lediglich die Unterbänder erfahren Lageveränderungen, wie das schon an anderer Stelle (1) an Hand der Photometerkurven geschildert wurde. In einem Teil der Fälle sind die Fibrillen mit einer indifferenten Substanz bedeckt, die die feineren Details verbirgt, jedoch die grosse Periode klar erkennen lässt. Abb. 2.

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THE EFFECT OF SOME COBALT PORPHYRINS IN THE MICROBIOLOGICAL ASSAY OF VITAMIN B_{12}

Z. G. BÁNHIDI, A. G. M. SJÖSTRÖM, and K.-G. PAUL

Division of Food Chemistry, The Royal Institute of Technology, Stockholm, and Medical Nobel Institute, Biochemical Department, Stockholm, Sweden

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Although it has not yet been conclusively demonstrated that vitamin B_{12} contains a tetrapyrrol structure, there are several observations supporting this assumption. Alkali fusion of B_{12} yields products which give a positive test for pyrrols (3). The spectrum of B_{12} exhibits a strong absorption band at around 360 mμ, the shape of which is very similar to that of a Soret band of a porphyrin (2, 5, 6). The wavelength shifts of this absorption band in ultraviolet light, and of the absorption band at around 550 mμ, upon the addition of cyanide are similar to the shifts in wavelength of the absorption bands of cobalt porphyrins upon the addition of various nitrogenous bases. Moreover, the intense red colour of B_{12} can scarcely be due only to its cobalt content. Thus 3 μg of B_{12} per ml gives a distinctly red colour while the equivalent amount of cobalt (0.12 μg) as nitrate does not visibly colour the solution red. The colour intensity of B_{12} is of the same order of magnitude as *e.g.* that of a porphyrin in neutral solution or of certain metalloporphyrins.

In the present investigation the microbiological activities of proto-, haemato-, and mesoporphyrins towards *Lactobacillus lactis* Dorner (A.T.C.C. 10697) and *Lactobacillus leichmannii 313* (A.T.C.C. 7830) were tested by means of the cup plate method. With the technique and the medium earlier described (1), growth was occasional and the reproducibility of the experiments was low. However, when the salt concentration was lowered and the nucleic acid derivatives and B-vitamins were replaced by a yeast autolysate, better growth and reproducible results were obtained.

Cobalt was introduced into the porphyrins as described by Fischer (4). Haematoporphyrin was obtained from its crystalline dihydrochloride, and the other two compounds by saponification of their dimethyl esters, previously purified by chromatography and crystallized from chloroform-methanol. The cobalt porphyrins were dissolved in a few drops of alcohol, and phosphate buffer of pH 7.0 was added to give final concentrations of 200, 100, and 50 μg cobalt porphyrin per ml. The final concentration of alcohol was about 1 per cent. Of the solutions to be examined 2–4 drops, depending upon the thickness of the agar plate, were added to the cup.

From the tables it can be seen that cobalt protoporphyrin and cobalt haematoporphyrin can stimulate the growth of *Lactobacillus lactis* Dorner or *Lactobacillus leichmannii* under the experimental conditions employed by us. The growth stimulating effect of cobalt protoporphyrin at the concentration used is in contrast to the inhibitory effect of protoporphyrin itself and of its iron complex. Cobalt mesoporphyrin does not stimulate but rather inhibits growth. This discrepancy may be due to the differences in the side chain arrangements of the three porphyrin molecules.

TABLE I

Concentration Substance	50 µg/ml	100 µg/ml	200 µg/ml
Co protoporphyrin	No effect	+	+ +
Co mesoporphyrin	No test	No test	— —
Co haematoporphyrin	No test	No test	+ + +
Co protoporphyrin + KCN	— —	— —	— — +[1]
Solvent + KCN	— —	— —	— —

TABLE I. Growth stimulating resp. inhibiting effects of cobalt porphyrins on *Lactobacillus lactis* Dorner. Cobalt protoporphyrin was also tested on *Lactobacillus leichmannii*, with the same results. + means growth, and — means inhibition around the cup. The number of signs is proportional to the diameter of the zone. The inhibition can be seen because of a faint, diffuse growth in the whole agar plate.

TABLE II

Microorganism Substance	*Lactobacillus leichmannii*	*Lactobacillus lactis* Dorner
Protoporphyrin	— —	— —
Ferri protoporphyrin chloride (haemin)	No effect	— — —
Co protoporphyrin	(+)	No effect
Solvent	No effect	No effect

TABLE II. Growth inhibiting effects of porphyrins. Concentration: 100 µg per ml. Symbols as for Table I. In the experiments of Table II heavier inoculates were used than earlier, and the resulting stronger, diffuse growth therefore masked the growth promoting effect of Co protoporphyrin towards LLD.

Protoporphyrin possesses two vinyl and haematoporphyrin two α-hydroxyethyl groups at the positions 2 and 4, while mesoporphyrin has the two less reactive ethyl groups at the same positions.

The cup plate method for the assay of vitamin B_{12} is more specific than the tube methods using liquid medium. With the cup plate method only desoxyribosides have so far been found to be substitutes for B_{12} as growth stimulators. The concentrations at which the two cobalt porphyrins stimulate growth are of the same order of magnitude as those at which the desoxyribosides stimulate, *i.e.* 200–400 times higher than for B_{12}. Their limited solubility in water and a possible effect of the agar medium upon the diffusibility of the cobalt porphyrins may explain why the growth zones do not have such distinct boundaries as those seen with B_{12}.

A detailed report will be published.

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[1] In this case a confined, distinct zone of growth appeared within the inhibition area.

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THE DETECTION OF NUCLEIC ACID IN TISSUES BY INFRA-RED MICROSPECTROMETRY

R. D. B. FRASER and J. CHAYEN

Wheatstone Physics Laboratory and Medical Research Council Biophysics Research Unit, King's College, London, Great Britain

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THE use of a characteristic visible or ultraviolet absorption band for the detection and estimation of cellular components has received much attention. In particular, the intense absorption band of the nucleic acids near 2 650 Å has been used by Caspersson (3) and others (4, 6) to follow the nucleic acid content and distribution, in individual cells, at various stages of growth. Although measurements of the infra-red spectra of tissue sections have been reported by Blout and Mellors (2) little advantage of the highly characteristic infra-red absorption bands has been taken. This is mainly due to the difficulties of assignment of the absorption bands, and the relatively large amounts of material required to obtain a spectrum.

By using a 0.8 N.A. reflecting microscope in conjunction with a Grubb-Parsons infra-red spectrometer (5), we have succeeded in obtaining spectra from small areas of a number of tissue sections. We were able to detect absorption bands between 850 and 1 000 cm^{-1} in these complex spectra which are known (1) to be characteristic of ribose and deoxyribose nucleic acids. In general, the infra-red absorption bands of cellular components below 1 300 cm^{-1} are very much weaker than those between 1 300 and 4 000 cm^{-1}. The nucleic acids, however, form an exception since the phosphate ester backbone of the molecules gives rise to intense absorption bands below 1 300 cm^{-1}.

Two examples of the way in which these assignments were confirmed are shown in figure 1. Spectra A and B were obtained from purified ribose and deoxyribose nucleic acids respectively. The full curve of spectrum C is that of a bean root tip section taken from the meristematic region. The characteristic ribose nucleic acid absorption bands at 860, 916, 969 and 997 cm^{-1} are clearly detectable. After extraction with ribonuclease these absorptions are greatly reduced, revealing absorptions due to other cellular components. Spectrum D shows how similar confirmation was obtained for the deoxyribose nucleic acid bands at 895, 930 and 967 cm^{-1}, in a smear of ram spermatozoa, after extraction with N/10 HCl.

It has been known for some time that purified ribose and deoxyribose nucleic acids could be differentiated on the basis of their infra-red spectra (1), but it is clear that this is also possible in substantially intact tissues, even though other materials are present in greater concentrations. Although quantitative estimations of concentration by infra-red absorption are difficult, there seems no reason why semi-quantitative information should not be obtained in cytochemical studies of nucleic acid content by this method. Certain progress in this respect has been achieved in the

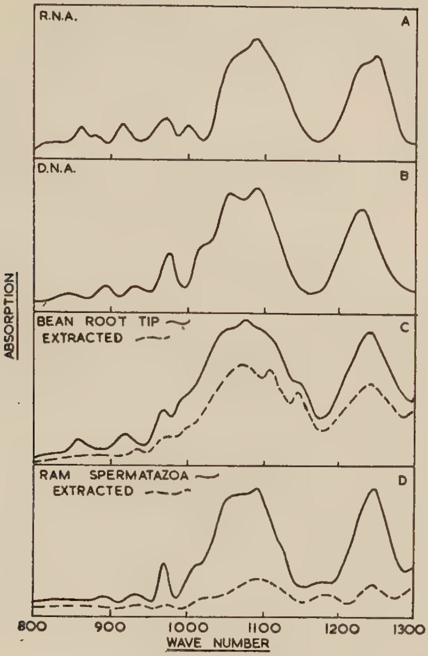

FIGURE. 1.

Infra-red absorption spectra.

A: Yeast ribose nucleic acid (sodium salt);
B: Thymus deoxyribose nucleic acid (sodium salt);
C: Bean root tip section, before and after extraction with ribonuclease;
D: Ram spermatozoa smear, before and after extraction with N/10 HCl.

estimation of the nucleic acid concentration in different regions of the bean root tip. Although it is unlikely that this technique will in any way replace the more refined ultraviolet methods, it has the important advantage that the two acids may be differentiated and estimated separately. Further, the infra-red spectra of mononucleotides are characteristically different from those of the polymerised and protein-bound nucleic acids, so that separate estimation of these should be possible.

Summary: Infra-red spectra have been obtained from small areas of tissue sections by the use of a reflecting microscope in conjunction with an infra-red spectrometer. Absorption bands characteristic of both types of nucleic acid are observed. The possibility of using these absorptions for their separate detection and estimation is discussed.

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INCORPORATION OF N^{15}-LABELED GLYCINE AND ALANINE INTO THE PROTEINS OF DEVELOPING SEA URCHIN EGGS

TORE HULTIN

The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

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Egg material: Eggs from the sea urchin *Psammechinus miliaris* (Gmelin) were used in the experiments.[1] Dilute suspensions of eggs were fertilized and allowed to develop at 22° C in glass trays as described by Lindahl (8). At least 90 per cent of the ripe eggs developed into normal plutei.

Isotope incubations: Equal samples were taken from the egg suspensions 1) just before fertilization, and 2) 0–24 hours after fertilization at 4 hour intervals. The samples were washed by centrifugation and then transferred to sea water containing the isotope. All samples were incubated with isotope for 4 hours. In the glycine experiments 1 mg of glycine (61 per cent N^{15}) in 5 ml of sea water (pH 8.0) was administered per 1 mg of total egg nitrogen. Higher glycine concentrations did not proportionally increase the rate of isotope incorporation (Fig. 1). In the alanine experiments a corresponding amount of DL-N^{15}-alanine, 2.4 mg, was supplied. All incubations were made with jelly-free eggs. After the incubations, the eggs were washed by centrifuging once in sea water and twice in isotonic NaCl-solution containing 0.05 volumes of isotonic sodium citrate solution. The eggs were frozen and stored at —20° C.

Preparation of protein samples: The frozen material was extracted 3 times with isotonic KCl-solution, and the soluble fraction as well as the residue were treated with 0.1 M carrier glycine or alanine for dilution of the free amino acid isotope. Both fractions were treated repeatedly with hot trichloroacetic acid and with lipid solvents. The nitrogen of the remaining, insoluble material was considered as protein-nitrogen. All extrations were made by grinding the material with an air-driven, rotating glass-rod.

Results: It seems likely that there is not an unrestricted, free diffusion of amino acids into the cells of unfertilized or developing sea urchin eggs. Moreover, changes in cell permeability have been demonstrated during the fertilization (11). Therefore it would be desirable to relate the incorporation of isotope into the cell proteins with the total amount of labeled amino acid, actually taken up by the cells in each single experiment. Unfortunately this latter value can scarcely be established since it seems very problematic to satisfactorily free the cells from traces of highly labeled amino acid trapped within the fertilization membrane space, blastocoel, etc.

Both in the glycine (Fig. 2) and alanine (Fig. 3) experiments the same general

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[1] The experiments were performed at the Zoological Station of Kristineberg, Sweden, during 1950–1951.

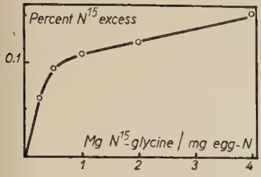

Fig. 1.

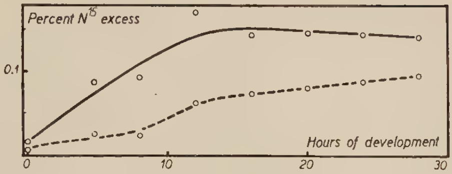

Fig. 2.

Fig. 1. Rates of isotope incorporation into the total protein nitrogen fraction after incubation of *Psammechinus* gastrulae for 4 hours with varying concentrations of N^{15}-glycine (12–16 hours after fertilization).

Fig. 2. Rates of isotope incorporation into the non-soluble (upper curve) and soluble (lower curve) cell proteins at different stages of development. The eggs were incubated with N^{15}-glycine for 4 hours. Mesenchyme cells appeared at 9 hours of development. Invagination started at 12 hours.

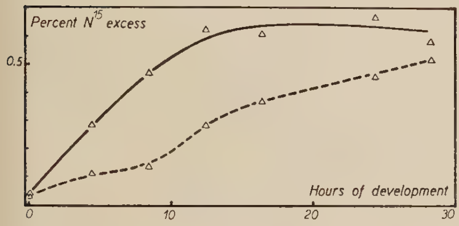

Fig. 3.

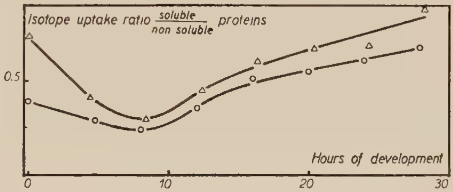

Fig. 4.

Fig. 3. Similar experiments as in Fig. 2 but isotope added in the form of N^{15}-DL-alanine.

Fig. 4. The isotope incorporation rates of the soluble proteins at different stages related to the corresponding incorporation rates of the non-soluble proteins. Upper curve: alanine experiments; lower curve: glycine experiments.

picture of isotope incorporation into the cell proteins during development was obtained. Sea urchin eggs contain large amounts of free glycine (7), which cause a considerable isotope dilution in glycine experiments. Probably also a higher metabolic activity of alanine contributes to the higher incorporation level found in the alanine experiments. In both glycine and alanine experiments the non-soluble proteins had a higher rate of incorporation than the soluble proteins. The rate of incorporation into the non-soluble proteins increased gradually soon after fertilization. After about 8 hours of development (blastula stage) the incorporation into the soluble proteins became intensified (Fig. 2, 3, 4).

The present results are in close agreement with previous experiments (5), where the incorporation of labeled ammonia into the proteins of developing *Paracentrotus* eggs was studied. As will be shown in a subsequent report, the isotope in such experiments is mainly incorporated into the protein amides. If it is assumed that granular cell elements (mitochondria, microsomes) are preferentially concentrated in the non-soluble protein fraction, and that prominent features of cell fluid proteins are revealed by the soluble fraction, the present results are well suited to the same interpretation that was suggested in the previous study (5): As a result of fertilization a progressive multiplication and growth (cf. 6) of small, granular cytoplasm constituents may be initiated. From the blastula stage onwards, such units become more and more capable of productive functions according to the actual demands of the differentiating organism. Labeled proteins are emitted (cf. 4) and appear in the cell fluid in increasing amounts (cf. 10). As will be shown in a later communication, other synthetic processes (syntheses of purines, pyrimidines and amino acids) are intensified during the same period. Increased enzymatic activities have also been reported to occur at the same time (9, 1, 2). Mitochondria reappear after the blastula stage in fertilized, mitochondria-free egg-quarters obtained by centrifugation (3).

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ANIMALIZING ACTION OF IODOSOBENZOIC ACID IN THE SEA URCHIN DEVELOPMENT

JOHN RUNNSTRÖM and GEORG KRISZAT

The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

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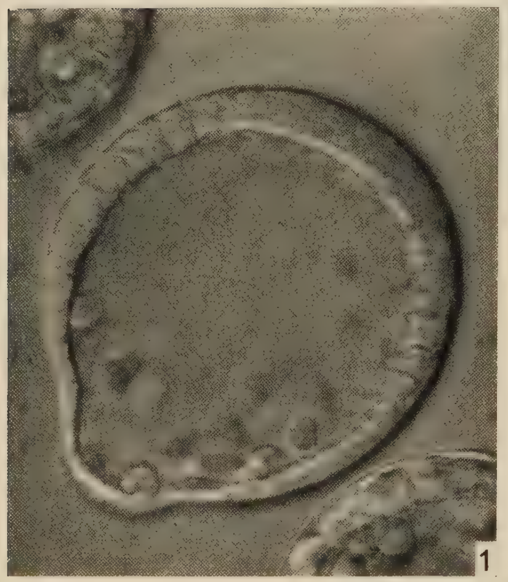
These writers have studied the effect of SH-reagents on the fertilization, cell division and development of the sea urchin egg (6, 11). In this connection the eggs were also exposed to various concentrations of iodosobenzoic acid (IBA) in sea water (pH~8). The effects on membrane formation and early development of *Arbacia punctulata* have been described elsewhere (9, 10). Some observations on the ensuing development will be presented below.

Eggs of *Arbacia punctulata* were transferred to I, sea water, II, $6.6 \cdot 10^{-4}$ M IBA in sea water. The fertilization and segmentation proceeded in a normal way, except for a slight delay in some of the eggs in II. *After 6 hours:* hatched blastulae in I, whereas no hatching in II, although the blastula stage is prevailing also here. *After 20 hours:* I, bilateral early plutei. II, 80–90 per cent of the larvae still within the membranes; even when hatching occurred the membranes did not dissolve, but remained visible on the bottom of the dish. The formation of the entoderm is suppressed in at least 90 per cent of the larvae. The animal region is characterized by a high cylindric epithelium that often could be shown to carry a ciliary tuft. This occupied a considerably larger area than in normal blastulae or gastrulae. The cells at the vegetal pole were flattened. In the interior a variable number of free cells were present. The larvae carried no pigment. The pigment originally present in the eggs had disappeared and no pigmented mesenchyme cells had appeared as in I. The larvae were obviously *animalized*, see (7). The free swimming larvae were or of the type described or had developed a small compact archenteron.

In the same experiment blastulae had been transferred 6 hours after fertilization from II to normal sea water (IIn). After 40 hours I contained plutei with well developed anal arms. In IIn 45 per cent of the larvae had a normal pluteus type. The rest of the larvae were less developed. The animal region was richer in cell elements than the more vegetal regions of the ectoderm. Anal arms were short or lacking, but the entoderm was normal. Single larvae were of a more pronounced animalized type: conical shape of the ectoderm with no arms; archenteron with fewer cells than normally and presenting no differentiation.

If the exposure to the IBA was extended beyond the 6–8 hours stage the reversibility of the effect diminished. To a variable extent the larvae retained an animalized type.

Similar experiments as those reported were carried out at a larger scale with *Psammechinus miliaris*. Fig. 1 represents a larva 21 hours after fertilization. The eggs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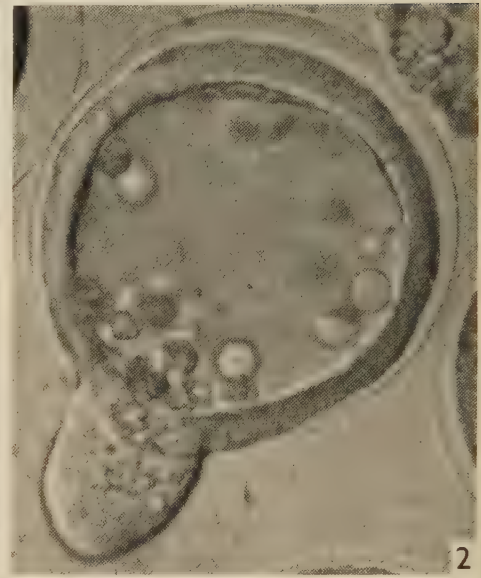
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were transferred to $1.3 . 10^{-3}$ M IBA in sea water 3 hours after fertilization. The larva of fig. 1 was still surrounded by the membrane. Like in *Arbacia* the breakdown of the membrane was inhibited in presence of IBA. The slightly bulging part of the larva with lower epithelium corresponds to the vegetal pole. A considerable displacement of cells from the vegetal pole could be observed to take place in IBA-larvae, thus making the vegetal region poorer in cells as compared with the animal region. This latter was constituted by high cylindrical cells. These larvae were transferred to normal sea water 22 hours 30 minutes after fertilization.

The control parallel to the larva represented in fig. 1 was in the "prism stage" (bilateral larva with fully invaginated archenteron, skeleton developed but no arms, cf. Hörstadius (5, p. 133)).

Fig. 2 shows a larva 9 hours after transfer to normal sea water (see above) from the same culture as that represented in fig. 1. Subsequent to the transfer a displacement of cells occurred in the direction of the vegetal pole, where now an entomesoderm appeared as a taplike protrusion. In this way exogastrulae were formed with an animalized ectoderm. The tuft of stiff cilia may occupy until 1/3 of the ectodermal region. The evaginated archenteron was injured to a variable extent with a marked gradient of injury in vegetal-animal direction. An invagination of the archenteron may also occur and a normally differentiated intestin arise. According to the time of exposure to IBA, all transitions may be obtained after transfer to normal sea water between animalized larvae with a small intestin to plutei of rather normal type. A considerable delay was always found with respect to the development of pigment cells and of a typical stomodeum. This will be described in detail elsewhere.

If the larvae were left in the IBA the entoderm or remained suppressed or a similar cell dislocation as that described above brought about the formation of a rudimentary entomesodermal region. The elements of the animal cell plate remained intact even after an exposure for 4–5 days in the IBA-solution. The cells were then very rich in mitochondria and the formation of the stiff cilia apparently went on continuously so that most impressive ciliary tufts arose. The more vegetal ectoderm or —if any—entomesoderm cells were strongly injured or altogether lysed at the same time—a striking case of differential susceptibility.

The above data tend to indicate that an oxidation of certain groups in particular of SH-groups by means of IBA suppresses the differentiation of the entomesoderm in the sea urchin development. The activation of oxidizable groups may be responsible for the more rapid reduction of red.-ox. dyes observed by Child (3) to occur at the vegetal pole of the late blastula stage. An increase in SH-groups may be an important factor in the differentiation of the entomesoderm as SH-groups are activators of many enzymes, see Barron (1). Furthermore SH-groups certainly play a great structural role in the cytoplasm.

Runnström and Thörnblom (12) contended that the vegetal differentiation was connected with a lower oxidation-reduction level than the animal differentiation. Lindahl *et al.* (8) assumed that the exposure of the unfertilized eggs to NaSCN brings about animalization by favoring the oxidation of certain groups in proteins, particularly of SH-groups. The data presented above tend to substantiate these ideas, see also Gustafson (4). There are many interesting analogies between the results outlined above and those obtained by Beatty (2) in a study on amphibian eggs subjected to the SH-reagent chloroacetophenone.

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THE EFFECT OF IODOACETAMIDE ON FERTILIZATION AND DIVISION OF THE SEA URCHIN EGG

GEORG KRISZAT and JOHN RUNNSTRÖM

The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

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According to Runnström (5), a normal membrane formation occurs in eggs of *Arbacia punctulata* inseminated in presence of e.g. 3.10^{-2} M iodoacetate. These studies on *Arbacia* eggs were taken up again, but this time the more easily penetrating iodoacetamide (IAA) was used. The results were, however, the same as previously (7). Upon insemination of eggs in presence of 10^{-4} M iodoacetamide a normal membrane formation occurred, even if the eggs had been exposed to the reagent for more than one hour prior to insemination. The complicated processes attending the membrane formation are thus not inhibited by the alkylating reagent. The ensuing processes are, however, strongly arrested. The nuclei remain in a peripheral position or at least no clear streak appears upon fertilization in presence of 10^{-4} iodacetamide. A still more potent reagent is the mercaptide forming p-chloromercuribenzoate (CMB). This reagent immobilizes the spermatozoa, but at appropriate concentrations a number of eggs may be fertilized before this occurs. A normal membrane formation may occur even when the migration and fusion of the nuclei are completely arrested. There is a high incidence of polyspermy in presence of CMB. A most striking phenomenon may occur upon fertilization in presence of or subsequent to pretreatment with CMB (concentration: $2.8 \cdot 10^{-5}$ M or higher). A strong contraction of the cortical layer occurs so as to squeeze out the interior cytoplasm in a varying number of pigmentfree lobes. The cortex containing all the pigment shrinks to a small folded sac. The phenomenon was considered to reflect the normal slight contraction of the egg surface occurring upon fertilization. A reaction that normally reverts the surface contraction is blocked in presence of the mercaptide forming, but not in that of the alkylating agent.

Brief reference will be made here to further experiments on the effect of IAA on the eggs of *Echinus esculentus* (*Ech.*) and *Psammechinus miliaris* (*Ps.*). The eggs of *Ech.* were kept at 10°, those of *Ps.* at 20°. The time for first division was 4 hours and 45–50 minutes respectively.

Unfertilized eggs of *Ech.* were immersed in 10^{-4} M IAA and inseminated 30 minutes later in presence of the agent. Membrane formation occurred as in the control, but the fusion of the nuclei failed in general to occur. A sample (I) of the eggs was transferred to normal sea water 30 minutes after fertilization. A spermaster developed with the radial strands more marked than in the control eggs. No division occurred; some per cent of the eggs reached the amphiaster stage and were elongated in the direction of the spindle, but stopped in this stage that under normal conditions immediately precedes the division. In addition to I the following samples were prepared:

Eggs were transferred to 10^{-4} M IAA 30 (II), 90 (III), 150 (IV) and 210 (V) minutes subsequent to fertilization. C designates a parallel control in sea water. After an exposure for 60 minutes, the eggs were transferred to normal sea water. 18 hours after fertilization the following observations were made:

C: 100 per cent early blastulae. I: No division, II: 90 per cent early blastulae, 10 per cent in earlier cleavage stage, III: Late cleavage stages, IV: 90 per cent early blastulae, 10 per cent 2-cell with numerous nuclei, V: Early blastulae.

The eggs of sample I have thus proved to be the most susceptible to the IAA-action. Additional experiments confirmed that the period of maximum susceptibility is that immediately following upon the fertilization. The unfertilized eggs always exhibited a much lesser susceptibility to IAA than the fertilized eggs.

Eggs of *Ps.* were subjected in different stages to 10^{-4} M IAA in sea water. After 20 minutes they were transferred to normal sea water and the further development of the following samples was observed: C untreated control. I. Immersed before fertilization, fertilized in normal sea water. II. Immersed immediately after fertilization. III. Immersed 20 minutes after fertilization. IV. Immersed 40 minutes after fertilization. After 19 hours the percentages of blastulae were the following: C: 100, I: 85, II: 0, III: 50, IV: 85.

In II the eggs were in early cleavage stages, sometimes with incomplete cytoplasmic division. The blastulae in I were similar to those in C, whereas the blastulae in III and IV were less advanced and less regular than those in C. The non blastulae in III and IV represented advanced cleavage stages.

The changed properties of the cortical layer upon fertilization is manifested when the eggs are immersed in hypertonic sea water (2 ml sea water + 0.6 ml 2.5 N NaCl); 5–10 minutes after fertilization the angular type of plasmolysis prevails, for references see (6). The angular plasmolysis is very different from the wrinkled plasmolysis of unfertilized eggs or the smooth plasmolysis exhibited by eggs immediately upon fertilization. Eggs were immersed in 10^{-3} or 10^{-4} IAA, and 30 minutes later fertilized in presence of the agent. After different intervals the *Ech.* eggs were transferred to the hypertonic medium. The IAA-treated eggs showed an angular plasmolysis like the fertilized control eggs. But in the former the maximum of angular plasmolysis was reached somewhat later, after $1\frac{1}{2}$–2 minutes as compared with 1 minute in the control. Furthermore the angular shape was less pronounced in the IAA-treated eggs than in the control. The smoothing of the control eggs occurring after 10–12 minutes is, on the other hand, somewhat slower in the IAA-treated eggs. The important structural change of the cortex manifested in the angular plasmolysis thus develops in presence of the alkylating agent. Neither does CMB inhibit the said structural change. Angular plasmolysis occurs also in concentrations of the agent that stop migration of the pronuclei.

The rigidity of the cytoplasm decreases under the influence of IAA. *Ech.* eggs were transferred 2 hours after fertilization to 10^{-4} M IAA. They were centrifuged half an hour later for 7 minutes at about 4 500 × g. A parallel control showed a slight tendency to displacement of the mitochondria toward the centrifugal egg pole. In the IAA treated eggs the mitochondria were accumulated as a dense layer at the centrifugal pole. Below the centripetal pole a non granular layer appeared extending to about 1/5–1/4 of the egg diameter along the axis of centrifugation. The eggs were somewhat elongated in this direction. The decrease in rigidity under the action of IAA constitutes a similarity with the effects produced by colchicine (1). Like colchicine IAA also brings about a shortening of the spindle. The stages of maximum susceptibility are, however, widely different as follows from a comparison of the above date with those presented by Beams and Evans (1).

The period of high sensitivity toward IAA (and CMB) prevailing immediately after fertilization coincides with the period of high glutathione content according to Shearer (8) and Infantellina and La Grutta (2). It must remain undecided whether a second maximum exists during the first mitotic cycle, as is to be expected in view of Rapkine's data (4). Particularly striking is the coincidence with the activation of an SH-enzyme of proteolytic nature demonstrated by Lundblad (3). This enzyme plays thus no role for the initiating cortical changes ensuing upon the attachment of the spermatozoon, but it may influence the subsequent processes.

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OBSERVATIONS ON THE EFFECT OF ESTRADIOL BENZOATE ON THE INCORPORATION OF RADIOACTIVE PHOSPHORUS IN THE VAGINA OF THE RAT

ULLA AMINOFF, ERIK ODEBLAD and KARL-HENRIK WETTERDAL

Women's Clinic of Karolinska Institutet, Sabbatsberg Hospital, Stockholm, Sweden

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THE present work is a report on the effect of estradiol benzoate on the accumulation of radioactive phosphorus in the vagina, studied with autoradiography.

Experimental: 8 adult female rats were used, originating from the same littermate and weighing about 200 g. The stock was inbred for more than 10 generations. They were oophorectomized bilaterally. Before operation, the animals presented regular estrus cycles. After operation the vaginal smears showed no signs of spontaneous estrogenic stimulation. 7 days after oophorectomy, 4 of the animals received 0.05 mg estradiol monobenzoate, dissolved in 0.3 ml arnot oil subcutaneously. The remaining 4 animals served as controls. 24 hours after the injection of the estrogenic hormone, the animals received 1 μC P^{32} as orthophosphate per g of body weight subcutaneously in a 1 per cent solution of Na_2HPO_4. The injected volume was about 1 ml.

8 hours later the animals were killed with ether, and the vagina was immediately removed and put in a 6 per cent solution of formalin, containing 0.5 per cent trichloracetic acid for fixing during 24 hours. Paraffin embedding and sectioning were then undertaken. 10 μ thick sections were made and mounted on methacrylate slides. They were then pressed against Gevaert Dentus Rapid film for 24 days, thus allowing

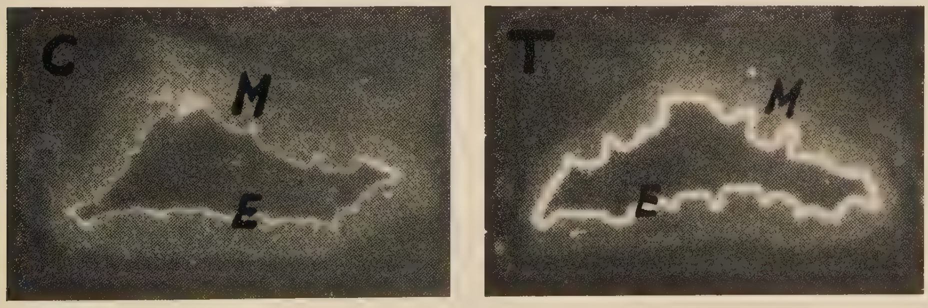

Fig. 1. Enlargements (10 times) of autoradiographs of vagina. C from control, T from estrogen-treated animal. E = epithelium, M = muscular layer. The difference between the epithelial linings on the two pictures is clearly visible.

the autoradiographic exposure. A standard light exposure was applied on a limited area of each film serving as a control of the development. This procedure was carried out in Kodak DK 20 with KBr added to 0.2 per cent for 30 minutes at +18.°3 C. The silver of the reverse side of the film was removed with Farmer's solution in excess. The histological sections were stained with haemalan-eosin. The mean autoradiographic resolution was about 20 μ (4).

The sections and the autoradiographs were examined qualitatively, using magnification of 42–310 times, and compared with each other. The autoradiographs were evaluated with the aid of a recording photometer (5). The photographic densities corresponding to the vaginal epithelium and muscular layer were calculated from the photometer records.

1. *Examination of vaginal smears.* In the control animals no cornified cells were visible in the smears. When the estrogen-treated animals were killed, large amounts of desquamated cornified cells appeared, indicating intense estrogenic stimulation.

2. *Examination of histological sections.* In the control animals the vaginal epithelium was thin, consisting of 2–4 layers of cells. Cornified cells were absent. In the estrogen-treated animals, the epithelium consisted of about 6–8 cell layers. In addition, a superficial layer of cornified cells was present.

In the muscular wall no appreciable differences between controls and estrogen-treated animals were visible.

3. *Qualitative examination of autoradiographs.* In both estrogen-treated animals and in controls, a dense line appeared, corresponding to the epithelium. The density appeared considerably greater for the estrogen-treated animals than for the controls.

In the area corresponding to the muscular layer, the density was considerably lower than for the epithelium and no appreciable difference was seen when comparing the estrogen-treated animals with the controls. Fig. 1 exemplifies these qualitative findings.

4. *Quantitative examination of autoradiographs.* Relative exposures from P^{32}, calculated from the densities of the epithelium and the muscular wall, are shown in Table I. Double determinations yielded the mean error 0.07 for 4 double determinations.

The mean relative values of the exposure for the *epithelium* were found 1.09 for estrogen-treated animals and 0.56 for controls. The difference, amounting to 0.53, is significant ($P < 0.01$).

The mean relative values for the *muscular wall* were found 0.41 for estrogen-treated animals and 0.32 for controls. The difference 0.09 is not significant.

Discussion: The histological treatment used causes a partial dissolution of acid-soluble and lipid-soluble compounds (5). The recorded amounts of P^{32} therefore belong to a great many biochemical components. Estrogens cause both hyperaemia and proliferative changes in the vaginal tissues (3). Further work (1) indicates increased labelling of easily hydrolysable acid-soluble phosphorus compounds after estrogenic stimulation in the rabbit. All these factors may contribute to the increased incorporation of P^{32} after estrogenic treatment, revealed by the present investigation.

The reaction upon hormonal stimulation found here may reflect a more general phenomenon, as similar reactions were found in other organs. Thus, the uptake of P^{32} in the uterine epithelium was found to redouble upon estrogenic stimulation (6). Treatment with ACTH causes the same effect in the adrenal cortex (2).

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AMYLASE IN BULL SPERMATOZOA

GUNNAR LUNDBLAD and ESKIL HULTIN

The Wenner-Gren Institute for Experimental Biology, and the Biochemical Institute, University of Stockholm, Sweden

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Various results obtained in fertilization experiments during the last few years have encouraged Runnström (3) to forward the idea that in the fertilization, the egg membrane, which probably contains some mucopolysaccharide, is partly hydrolyzed by the sperm carbohydrases. It has recently been shown by these writers (2) that carbohydrases are present in the spermatozoa of some evertebrates (*Psammechinus, Modiola*), and we have now tested bull spermatozoa for amylase and cellulase activity.

Experimental. 8.0 ml bull sperma were diluted with 25 ml Ringer solution and the spermatozoa centrifuged off. They were washed twice with 25 ml Ringer solution (without losing their motility) and finally suspended in 20 ml distilled water for cytolysis for 15 hours. The suspension was frozen, ground and thawed up. After the extract was centrifuged, its activity was determined.

For the amylase determination 2 ml 0.82 per cent starch solution, 1 ml buffer solution (acetate or phosphate) and 1 ml extract were mixed, and the breakdown of the starch was followed viscosimetrically. The activity calculations were made as previously described (1). The results given in the graph indicate that the amylase has its optimum activity at pH about 7. The amylase activity of the wash fluids was also measured and was considerably lower than that of the extract.

The activity of the extract at 35.5° C and optimum pH was 85 mμA/ml, and the nitrogen content was 0.10 mg N/ml. Using a conversion factor previously determined (1) we get the following figure for the activity of the amylase at optimum pH in relation to the nitrogen content: 85×10^{-6} millieqv. reducing sugars/min. × mg N.

For the test for cellulase activity we used hydroxyethylcellulose ether. However, the reaction mixture showed no significant drop in viscosity.

Conclusion. It is plausible to presume that the mucopolysaccharide of the egg membrane is not the same for all animals. The determination of the structure of its carbohydrate part will, of course, be a very difficult task. However, by investigating which carbohydrases are present in the spermatozoa of an animal, one gets some information about the nature of the membrane of the corresponding egg, for such carbohydrate linkages as cannot be hydrolyzed by the enzymes in the spermatozoa cannot be of any prime importance in fertilization. Whereas we previously reported (2) the presence of not only amylase but also cellulase in the spermatozoa of a sea urchin—which indicates that both α- and β-1,4-linkages between glucose residues in the egg membrane of the sea urchin may be (but are not necessarily) of importance in its fertilization—, we have not found cellulase in bull spermatozoa, although we

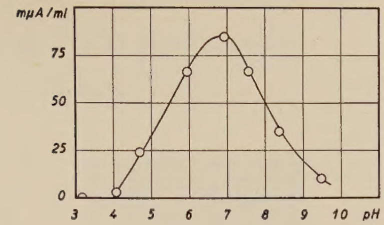

Fig. 1. The amylase activity of a bull spermatozoa extract versus pH.

found amylase. Therefrom we conclude that α-1,4-linkages between glucose residues in the egg membrane of the cow may be of importance in its fertilization, but that β-1,4-linkages between glucose residues, situated in a similar manner as in cellulose, either are not present or are of no importance.

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